Studies on the regulatory mechanism of

the ULK1 complex in the induction of autophagy

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Dedication

I dedicate this thesis to the BMBB program at the University of Minnesota.

Abstract

Autophagy, an evolutionarily-conserved cellular process through which organelles and macromolecules are degraded in the lysosome, is induced under nutrient starvation or other unfavorable growth conditions. Unc51-like kinase 1 (ULK1) is a serine/threonine protein kinase that plays a key role in the autophagy induction process, but how ULK1 is regulated by cellular signals for induction of autophagy and how ULK1 regulates the downstream processes in autophagy remain poorly understood. ULK1 interacts with Atg13, focal adhesion kinase family interacting protein of 200 kD (FIP200) and Atg101 to form a large protein complex involved in early steps of the autophagy induction process. To better understand the function of the ULK1 complex, my thesis work has sought to identify binding proteins of the complex. Through a yeast two hybrid screen using a human fetal brain cDNA library with Atg13 as bait, a protein named MCF.2 cell line derived transforming sequence-like 2 (MCF2L2) was identified. Through co-immunoprecipitation and *in vitro* binding assay, MCF2L2 was determined to directly interact with Atg13 via its N-terminal region independently of ULK1. Knockdown of MCF2L2 inhibited the formation of autophagosome and autophagy flux and led to accumulation of p62/sequestosome-1, a protein degraded through autophagy. Knockdown of MCF2L2 also suppressed the aggregation of WD-repeat protein interacting with phosphoinositides-1, an autophagic isolation membrane marker. MCF2L2 contains a putative Rho-guanine nucleotide exchange factor (GEF) domain in the middle and has a sequence similarity to MCF2L and MCF2, the well-known Rho-GEFs. MCF2L2 overexpression induced a moderate increase in the active forms of Rho GTPases and MCF2L2 colocalized with actin related protein 3, the actin nucleation factor that is regulated by Rho GTPases, implying that MCF2L2 potentially contains GEF activity. MCF2L2 knockdown partially suppressed the distribution of Atg9 from trans-golgi network to the cytoplasm in response to starvation, a process that may depend on actin cytoskeleton. Combined, these results suggest that MCF2L2, as a component of the

ULK1 complex, might play an important role in mediating signal transduction between the actin cytoskeleton and autophagy induction.

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List of Abbreviations

AIDS, acquired immune deficiency syndrome
ALD, alcoholic liver disease
AMBRA1, activating molecule in Beclin 1-regulated autophagy-1
AMPK, 5' AMP-activated protein kinase
ARHGEF, Rho guanine nucleotide exchange factor
Arp2/Arp3, actin related protein 2/actin related protein 3
ATZ, alpha1-antitrypsin Z
Bif, Bax-interacting factor
CaMKK, calcium calmodulin-dependent protein kinase kinase
CMA, chaperone-mediated autophagy
CI-MPR--cation independent mannose-6-phosphate receptor
DAPK, death-associated protein kinase 1
Dbs, Dbl's big sister
DFCP-1, double FYVE-containing protein-1
DMSO, dimethyl sulfoxide
ER, endoplasmic reticulum
FIP200 , focal adhesion kinase family interacting protein of 200 kDa
GAP, GTPase activating protein
GAPDH, glyceraldehydes-3-phosphate dehydrogenas
GATE16, golgi-associated ATPase enhancer of 16 kDa
GABARAP, gamma-aminobutyric acid receptor-associated protein
GEF, guanine nucleotide exchange factor
GSH, glutathione
GST, glutathione S-transferase
hVps34, human vacuolar protein sorting 34 homologue
IPTG, isopropyl-1-thio-β-galatopyranoside
IRS-1, insulin receptor substrate-1
JMY, junction-mediating regulatory protein
LC3, microtubule-associated protein1 light chain 3
LKB1, liver kinase B1
LI, lysosomal inhibitors
MAP4K, mitogen-activated protein 4-kinase
MCF2L2, MCF.2 cell line derived transforming sequence-like 2
MEFs, mouse embryonic fibrablasts
MHC, major histocompatibility complex
MLC, myosin light chain
mTOR, mammalian target of rapamycin
mTORC1, mammalian target of rapamycin complex 1
NPF, nucleation-promoting factors
p62, sequestosome 1

PA-PLA, phosphatidic acid-preferring phospholipase A
PCOS, polycystic ovary syndrome
PDK, 3'-phosphoinositide dependent protein kinase
PE, phosphatidylethanolamine
PI3KC3, class III phosphatidylinositol-3-kinase
PI3P, produces phosphatidylinositol-3-phosphate
PIP_2, phosphatidylinositol (4,5)-bisphosphate
PIP_3, phosphatidylinositol (3,4,5)-triphosphate
PKA, protein kinase A
PRAS40, proline-rich Akt substrate 40 kDa
PTEN, phosphatase and tensin homolog
Raptor , regulatory-associated protein of mTOR
REDD, regulated in development and DNA damage responses
Rictor, raptor-independent companion of mTOR
ROCK, Rho-associated kinase
Rubicon, RUN domain and cysteine-rich domain containing Beclin-1-interacting protein
S6K, S6 kinase
SQSTM1, sequestosome 1
SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors
Snf, sucrose non-fermenting
SNP, single nucleotide polymorphism
SKD, suppressor of K(+) transport growth defect
STK, serine/threonine kinase
TCA, tricarboxylic acid cycle
Tecpr, tectonin domain-containing protein
TGN, trans-Golgi network
TLR, toll-like-receptor
TSC2, tuberous sclerosis complex 2
ULK1, Unc51-like kinase 1
Unc-51, uncoordinated-51
UVRAG, UV radiation resistance-associated gene
VAMP, vesicle-associated membrane protein
WASH, WASP/Scar homolog
WASP, Wiskott-Aldrich Syndrome protein
WAVE, WASP-family verprolin homolog
WHAMM, WASP homolog associated with actin, membranes and microtubules
WH2, WASP-homology-2
WIPI, WD-repeat protein interacting with phosphoinositides
ZIPK, zipper-interacting protein kinase

CHAPTER 1. BACKGROUND

This background section will discuss the protein components and protein complexes that are involved in the autophagy processes and how they function in the pathway. Several key protein complexes, including the unc51 like kinase 1 (ULK1) complex and the Beclin 1 complexes, will be discussed with respect to their compositions and functions. This section will also summarize the current knowledge about the cellular and physiological functions of autophagy. The role of ULK1 complex in autophagy will be reviewed and at the end, I will discuss about the missing gaps in our knowledge about the autophagy processes and how I have shaped my thesis work based on the missing gaps.

I. The autophagy machinery

Autophagy is a catabolic process evolutionarily conserved in eukaryotes through which cytoplasmic contents are delivered to the lysosome for degradation. There are four major types of autophagy; chaperone-mediated autophagy (CMA), microautophagy, macroautophagy and selective autophagy (Mizushima, 2009) (Figure 1). CMA is mediated by chaperone proteins that recruit target substrates to receptors on the lysosome for subsequent unfolding and degradation (Li et al., 2011a). Microautophagy is a membrane process of invagination, protrusion or separation of the lysosomal membrane through which cytoplasmic materials are engulfed directly by the lysosome (Li et al., 2011b). Macroautophagy is a lysosomal degradation of cytoplasmic components or compartments captured in a double membrane structure (Eskelinen, 2005; Xie and Klionsky, 2007). The double-membrane vesicular structure called autophagosome is the hallmark developed in the beginning of macroautophagy. While macroautophagy is considered a process of random engulfment of cytoplasmic components, an autophagic

process through which damaged organelles or extrinsic pathogens are selectively recognized and degraded in the lysosomes has been identified and called selective autophagy (Narendra et al., 2008; Thurston et al., 2009). Depending on what contents are recognized and degraded, selective autophagy is further divided into several types. For example, the selective autophagy process to eliminate damaged mitochondria is called mitophagy (Geisler et al., 2010; Kanki et al., 2009; Narendra et al., 2008). The processes to eliminate peroxisome (Iwata et al., 2006; Kim et al., 2008), mature ribosome (Kraft et al., 2008), endoplasmic reticulum (ER) (Bernales et al., 2006), or invading pathogens (Thurston et al., 2009; Yoshikawa et al., 2009; Zheng et al., 2009b) are called pexophagy, ribophagey, reticulophagy, or xenophagy, respectively. Although the selective autophagy depends on adaptor molecules in recognition of target organelles and macromolecules, the process shares many components with macroautophagy for the degradation steps (reviewed in (Weidberg et al., 2011)).

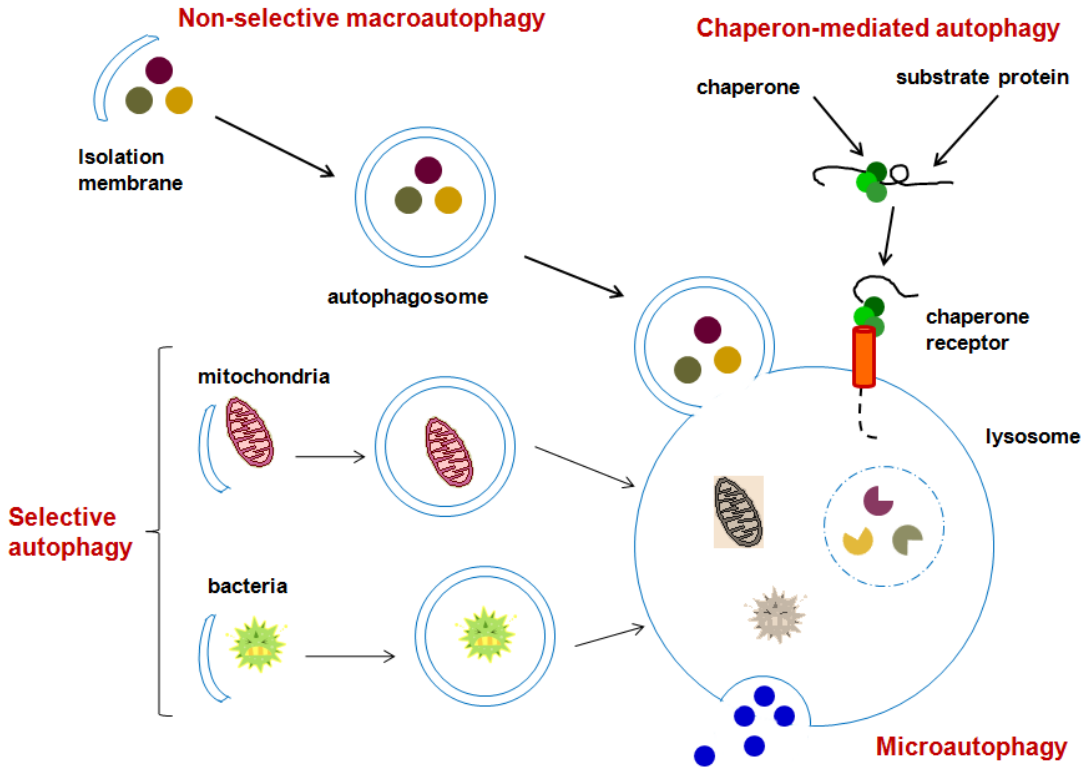


Figure 1. Depiction of major forms of autophagy.

The major forms of autophagy are illustrated. Counterclockwise from bottom right: Microautophagy is a process where cytoplasmic materials are engulfed directly into the lysosome (Li et al., 2011b). CMA is mediated by chaperone proteins that recruit substrate proteins to receptors on the lysosome for unfolding and degradation (Li et al., 2011a). Non-selective macroautophagy starts from the generation of the double layer isolation membrane, which expands to sequester cytosolic contents and encloses to form autophagosome that eventually fuses with lysosome (Eskelinen, 2005; Xie and Klionsky, 2007). Selective autophagy specifically targets certain organelles or pathogens for autophagosome-dependent transport and lysosome-dependent degradation (Narendra et al., 2008; Thurston et al., 2009).

Macroautophagy is the most widely studied and best characterized process compared to other autophagy processes. My thesis work has mainly focused on macroautophagy, although the cellular machinery of my study would also be important for selective autophagy, since macroautophagy and selective autophagy share many proteins in common during the processes (Kim et al., 2008; Komatsu et al., 2007; Zheng et al., 2009b). The process of macroautophagy can be divided into five subsequent steps (Mizushima, 2009): (1) formation of isolation membrane (the membrane structure initially generated upon autophagy induction (2) expansion of the membrane structures in size; (3) maturation of the membrane vesicle to form the circular double membrane structure called autophagosome; (4) membrane fusion of autophagosome with the lysosome; (5) degradation of the autopahgosomal contents in the lysosome (Figure 2). Below is a brief discussion about each of those steps.

(1) Formation of the isolation membrane: The autophagy process is initiated with the formation of a membrane compartment termed phagophore or isolation membrane (Militello and Colombo, 2011; Mizushima et al., 2001). The membrane structure has been proposed to originate from multiple sources such as the ER (Hayashi-Nishino et al., 2009; Yla-Anttila et al., 2009), mitochondria (Hailey et al., 2010), Golgi (Geng et al., 2010; Nair et al., 2011), trans-golgi network (TGN) (Young et al., 2006) and plasma membrane (Moreau et al., 2011; Ravikumar et al., 2010). The ER has been considered as the major source of the membrane. In a proposed model, an "Ω"-shaped membrane structure or "omegasome" is formed on the surface of the ER (Axe et al., 2008; Hayashi-Nishino et al., 2009). Omegasome was proposed to function as a platform for the formation of the isolation membrane (Hayashi-Nishino et al., 2009; Mari et al., 2011; Yoshimori and Noda, 2008). The molecular mechanisms underlying the formation of the isolation membrane remain poorly understood. Recent genetic and biochemical studies have shown that Atg1/ULK1 protein kinase is important for the formation of the isolation membrane (Chan et al., 2007; Ganley et al., 2009; Hosokawa et al., 2009a; Itakura and Mizushima, 2010; Kuroyanagi et al., 1998; Yan et al., 1998; Young et al., 2006).

The formation of the isolation membrane also requires Beclin 1 and its associated proteins. Beclin 1, the mammalian orthologue of yeast Atg6, interacts with hVps34 (human vacuolar protein sorting 34 homologue), a lipid kinase that is also called PI3KC3 (class III phosphatidylinositol-3-kinase) (Funderburk et al., 2010; Itakura et al., 2008; Liang et al., 2008; Matsunaga et al., 2009). Beclin 1 stimulates the lipid kinase activity of hVps34 to recruit additional Atg proteins (Wirawan et al., 2012). Beclin 1 and hVps34 interact with Atg14L, a key molecule involved in the formation of the isolation membrane (Itakura et al., 2008; Matsunaga et al., 2010; Matsunaga et al., 2009), The hVps34 protein complex containing Atg14L catalyzes the phosphorylation of phosphatidylinositol (PI) to produce phosphatidylinositol-3-phosphate (PI3P). PI3P recruits proteins containing the Fab1p/YOTB/Vac1p/EEA1 (FYVE) motif, such as double FYVE-containing protein 1 (DFCP1), WD-repeat domain phosphoinositide-interacting protein-1 (WIPI-1) and WIPI-2 (Polson et al., 2010). DFCP1, which is distributed diffusely throughout the ER or the Golgi, is re-distributed onto omegasome in response to starvation (Itakura and Mizushima, 2010; Matsunaga et al., 2010; Polson et al., 2010). WIPIs also translocate to the site of isolation membrane formation upon autophagy induction (Itakura and Mizushima, 2010; Polson et al., 2010). The recruitment of DFCP and WIPIs is crucial for the formation of the isolation membrane (Matsunaga et al., 2010; Polson et al., 2010). The formation of isolation membrane also depends on an ER membrane protein called vacuole membrane protein 1 (VMP1) (Ropolo et al., 2007). Through acquisition of extra lipids, the isolation membrane further expands and engulfs a portion of cytoplasmic material targeted for destruction to form the nascent double-membrane autophagosome (Itakura and Mizushima, 2010).

(2) Elongation of double membrane structure. The elongation step of the isolation membrane requires two ubiquitin-like conjugation systems. The first is the Atg12–Atg5 conjugation, which is produced by Atg7 (E1-like) and Atg10 (E2-like) enzymes. Atg12 is conjugated to Atg5 and these two proteins form a protein complex with Atg16L (Fujita et al., 2008). The Atg16L-Atg12-Atg5 complex is localized to the isolation membrane and plays an essential role for the membrane

expansion (Xie and Klionsky, 2007). This membrane expansion process requires fusion of membrane that depends on the soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) proteins such as vesicle-associated membrane protein 7 (VAMP7) and its partners (Moreau et al., 2011). The second conjugation system involves the phosphatidylethanolamine (PE)-conjugated Atg8 proteins (Xie and Klionsky, 2007). In mammalian cells, several homologues of Atg8 have been identified. They are microtubule-associated protein 1 light chain 3A and B (LC3A and LC3B), Golgi-associated ATPase enhancer of 16 kDa (GATE16), gamma-aminobutyric acid receptor-associated protein (GABARAP), and GABARAP-like protein (GABARAPL or Atg8L). The PE-conjugation of Atgt8 homologues is mediated by Atg7 and Atg3 (E2-like) enzymes (Nakatogawa et al., 2009; Weidberg et al., 2010). Atg3 and Atg12 recruit LC3 close to PE in the membrane. The Atg16L-Atg12-Atg5 complex provides the scaffold for LC3 to transfer LC3 from Atg3 to PE, and the lipidation of LC3 propels membrane elongation (Fujita et al., 2008).

Another key molecule involved in the membrane elongation process is Atg9, a multi-spanning transmembrane protein. Atg9 resides in a juxta-nuclear region that contains the TGN and peripheral late endosomes and it plays an important role in sequestrating membrane for autophagosom (Saitoh et al., 2009; Young et al., 2006). (Young et al., 2006). Atg9 was found to undergo trafficking between the TGN, Rab9- and Rab7-positive endosomes and the LC3-positive autophagosome precursor membranes in response to starvation. This process is important for the formation and elongation of the isolation membrane (Webber and Tooze, 2010). It has been shown that the trafficking of Atg9 requires the activity of ULK1 and appears to depend on hVps34 (Young et al., 2006).

(3) Maturation of the double membrane structure: The inner and outer bilayer membranes eventually fuse to have one inside the other as two distinct and enclosed membranes, and this marks the maturation of the double-membrane vesicle called autophagosome. After closure of autophagosome, it undergoes maturation process by

fusion events with early and late endosomal vesicles prior to fusion with the lysosomes (Chua et al., 2011; Liang et al., 2008; Peplowska et al., 2008). hVps34 is involved not only in the early step of the isolation membrane formation but also in the step of autopahgosome maturation. hVps34 interacts with UVRAG (UV radiation resistance associated gene) and the complex regulates the autophagosome maturation through recruitment and activation of Rab7 GTPase via its direct interaction with C-VPS/HOPS complex (class C vacuolar protein sorting/homotypic vacuole fusion protein sorting) (Liang et al., 2008; Peplowska et al., 2008; Sun et al., 2010). The PI3KC3-UVRAG-HOPS complex also interacts with Rubicon (RUN domain and cysteine-rich domain containing Beclin-1-interacting protein) via UVRAG (Matsunaga et al., 2009; Zhong et al., 2009). Rubicon is an inhibitor of UVRAG thus negatively acting in the autophagosome maturation process (Matsunaga et al., 2009; Sun et al., 2010; Zhong et al., 2009). Moreover, the hVps34 complex interacts with AMBRA 1 (activating molecule in Beclin 1-regulated autophagy-1) (Fimia et al., 2007) and Rab5 (Ravikumar et al., 2008). The maturation of the autophagosome is also regulated by other factors such as the ATPase suppressor of K(+) transport growth defect-1 (SKD1) and the cytoskeleton (Kang et al., 2011).

(4) Fusion of autophagosome with the lysosome: Autophagosomes fuse with lysosomes (or vacuoles in yeast) to form autolysosomes, in which the cytoplasmic cargo is degraded by lysosomal hydrolases. Two SNARE proteins, Vti1b and VAMP8, mediate the fusion of autophagosomes with lysosomes (Furuta et al., 2010; Nair et al., 2011). Vti1b is derived from autophagic compartments, whereas VAMP8 originates from lysosomes. Knockdown of those SNAREs inhibited the fusion of autophagosomes with lysosomes, thereby disturbing autophagosomal fusion with lysosomes (Furuta et al., 2010; Nair et al., 2011). Tectonin domain-containing protein (Tecpr1) was also shown to promote autophagosome-lysosome fusion (Chen et al., 2012). Tecpr1 was originally found to be necessary for selective autophagic targeting of bacteria pathogens (Ogawa et al., 2011), and it interacts with Atg5 and WIPI-2 (Behrends et al., 2010). Later, it was

discovered that Tecpr1 localizes to autolysosome membrane and recruits Atg5. The binding of Tecpr1 to the Atg12-Atg5 conjugate prohibits Atg16L from binding, and further recruits PI3P. The connection of Tecpr1 with Atg12-Atg5 and PI3P promotes the specific fusion between autophagosomes and lysosomes (Chen et al., 2012).

(5) Degradation of delivered content. The organelles and macromolecules delivered by autophagosome are degraded in the lysosome. The lysosome contains hydrolyzing enzymes that break down proteins, lipids and carbohydrates. The resulting degradation products are then released back to the cytoplasm through the activity of membrane permeases and are recycled as cellular building blocks (Eskelinen, 2005). The degradation of autolysosomal products is also necessary for the attenuation of autophagy and the reformation of functional lysosomes (Yu et al., 2010). Many human diseases, such as Hurler syndrome, Gaucher disease, Tay-Sachs disease, Sanfilippo syndrome and Hunter syndrome, are associated with defects in lysosomal digestive functions (reviewed in (Winchester et al., 2000)).

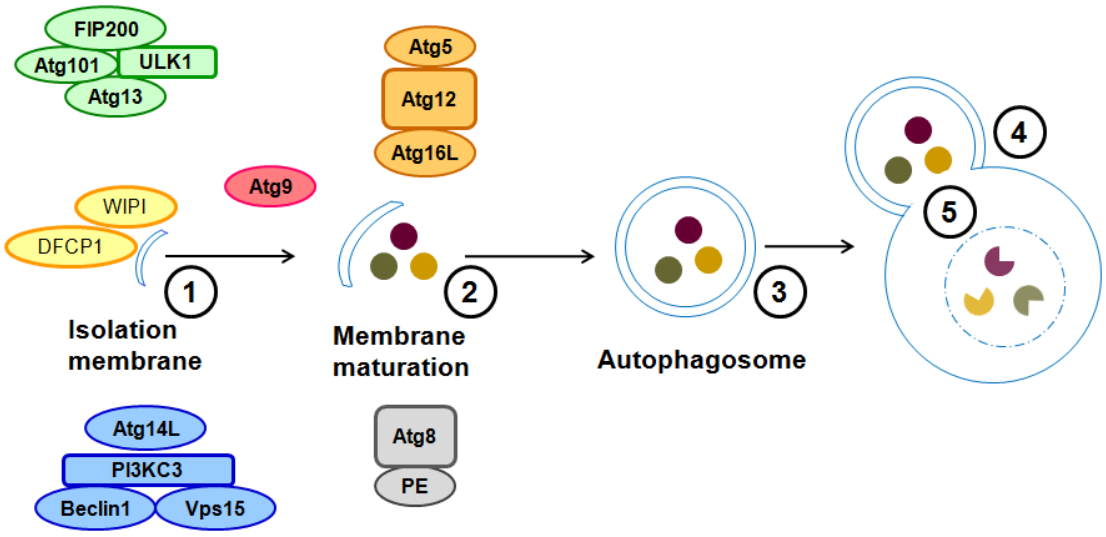


Figure 2. Illustration of the major stages in autophagy. The autophagy process can be divided into several stages. (1) Formation of the isolation membrane. The ULK1 complex and Beclin 1-associated class III phosphatidylinositol-3-kinase (PI3KC3) complex regulate the formation of the isolation membrane. PI3KC3 catalyzes the conversion of phosphatidylinositol (PI) to phosphatidylinositol-3-phosphate (PI3P), which is recognized by double FYVE-containing protein 1 (DFCP1) and WD-repeat domain phosphoinositide-interacting protein (WIPI). The recruitment of DFCP and WIPI is crucial for the formation of the isolation membrane. (2) Elongation of the isolation membrane. Two ubiquitin-like conjugation systems are required in this process. The first is the Atg12–Atg5 conjugation, and these two proteins form a complex with Atg16L. The second conjugation system involves the phosphatidylethanolamine (PE) conjugation of Atg8 proteins. There is another multi-spanning transmembrane protein Atg9, involved in the double membrane elongation. Atg9 undergoes trafficking between the TGN, Rab9- and Rab7-positive endosomes and the LC3-positive autophagosome precursor membranes in response to starvation to provide membrane source for autophagy.(3) Maturation of double membrane structure. The inner and outer bilayer membranes

eventually fuse to have one inside the other as two distinct and enclosed membranes, and this marks the maturation of the double-membrane vesicle called autophagosome. PI3KC3 is involved in the step of autopahgosome maturation. (4) Fusion of autophagosome with the lysosome: Autophagosomes fuse with lysosomes to form autolysosomes, in which the cytoplasmic cargos are degraded by lysosomal hydrolases. (5) Degradation of delivered content. The organelles and macromolecules delivered by autophagosome are degraded in the lysosome.

II. Broad roles of autophagy in physiology and diseases

Eukaryotic cells utilize autophagy to maintain the balance between the synthesis and degradation of macromolecules and organelles in response to nutrient deprivation, the availability of growth factors, and stress. Autophagy may contribute to cell survival under starvation by re-distributing nutrients for the most essential processes for cellular functions. As a key cellular process in response to environmental changes, autophagy is implicated in a number of physiological and pathological processes. In addition to its fundamental role in starvation adaption, autophagy is also important for embryo development, tissue differentiation, protection against neurodegeneration, pathogen processing, and cancer (Levine et al.; Mizushima, 2009; Rubinsztein, 2006). The following sections summarize the main functions of autophagy involved in physiology and human diseases.

a. Roles in physiology, development and differentiation

Energy metabolism

In a study using the mouse model, autophagy was up-regulated in the liver, pancreas, kidney, skeletal muscle, and heart within 24 hours of starvation (Mizushima et al., 2004). The cellular contents digested by the autophagy process can be used for cellular energy, and the liver can convert nucleotides, amino acids, and lipids into glucose and ketone bodies that are distributed to other parts of the body (Rabinowitz and White, 2010). Mammalian cells in the body usually live in a relatively constant nutrient environment. Nevertheless, autophagy is activated in the liver between meals to maintain its metabolic function (Mizushima and Klionsky, 2007). Even when the nutrient intake is sufficient, a basal level of autophagy operates for housekeeping and homeostasis (Mizushima and Klionsky, 2007). Autophagy has also been shown to regulate the breakdown of lipid droplets in the liver (Dong and Czaja, 2011; Singh et al., 2009a).

This alternative mechanism for lipid hydrolysis further highlights the significant function of autophagy in energy metabolism. Lymphocytes rely on growth factor stimulation to consume extracellular nutrients, so the energy metabolism in lymphocytes is sensitive to not only nutrient supply but also to fluctuation in growth factor levels (Hubbard et al., 2010; Mathew et al., 2007). Autophagy is activated in lymphocytes when growth factors are deprived, and this appears to function to accommodate the bioenergetic need of lymphocytes.

Development

Autophagy is observed as an early event in fertilized oocytes and is essential during the pre-implantation phase (Tsukamoto et al., 2008). Autophagy is massively induced in undifferentiated oocytes within 4 hours of fertilization (Tsukamoto et al., 2008). The mouse embryo cannot survive through the 4-cell to 8-cell stage if both the inherited maternal Atg5 and the oocyte specific *Atg5* are eliminated. *Atg5* knockout in mice does not result in early embryonic lethality but these *Atg5*-/- mice exhibit abnormality in embryonic neuron ubiquitin inclusion body and reduction in neonate adipose tissue mass (Tsukamoto et al., 2008). In late embryogenesis, a high level of autophagy is found only in limited tissues like the developing thymus (Mizushima et al., 2004), suggesting a tissue-specific role of autophagy in the development. For fetus of vertebrates, the sufficient nutrient supply during embryo development is suddenly terminated at birth. Autophagy is broadly activated in tissues except in the brain within 24 to 48 hours after birth to enable the neonates to survive the severe starved condition (Kuma et al., 2004). Mice with knockout of *Atg3* (Sou et al., 2008), *Atg5* (Kuma et al., 2004), *Atg7* (Komatsu et al., 2005), *Atg9* (Saitoh et al., 2009) or *Atg16L* (Saitoh et al., 2008) appear normal at birth but die within 1 day, supporting the indispensable role of autophagy at this stage.

Differentiation

During erythroid differentiation, erythroblasts release nuclei and degrade organelles before they are transformed into mature erythrocytes (Geminard et al., 2002). Mitochondrial clearance occurs during this process and it depends on autophagy. *ULK1* knockout mice showed impaired clearance of mitochondria in reticulocytes (Kundu et al., 2008). Mice with haematopoietic cell-specific knockout of *Atg7* accumulated damaged mitochondria in reticulocytes (Zhang et al., 2009a). These reticulocytes undergo apoptosis and the mouse usually die at 8-14 weeks due to severe anemia (Zhang et al., 2009a).

The haematopoietic cell-specific *Atg7* knockout mice mentioned above also have significant reduction in the numbers of T and B lymphocytes (Mortensen et al., 2010), possibly due to the increased cell apoptosis as a result of defective mitohagy. During the differentiation of T and B cells, mitochondrial content also goes through autophagic degradation (Pua et al., 2009). Lymphocyte-specific knockout of *Atg5* or *Atg7* in mice resulted in accumulation of mitochondria and subsequent increase in superoxide, making the cell more prone to apoptosis (Pua et al., 2009; Stephenson et al., 2009). This result suggests that autophagy is in charge of the clearance of mitochondria during the differentiation of T and B cells (Pua et al., 2009).

Pre-adipocytes differentiate into adipocytes with a large accumulation of lipid droplets. Knockdown of *Atg7* or knockout of *Atg5* in 3T3-L1 pre-adipocytes or in mouse embryonic fibrablasts (MEFs) decreased the accumulation of triglycerides and suppressed adipocyte differentiation (Baerga et al., 2009; Singh et al., 2009b). Adipocyte-specific knockout of *Atg7* in mice has resulted in reduction of the white adipose tissue mass. The reduction was accompanied with smaller sizes of adipocytes, increased mitochondrial content and lipid droplets, which are representative characteristics of brown adipocytes (Singh et al., 2009b; Zhang et al., 2009b). Consistent with these cellular phenotypic changes, the adipocytes in the mutant mice expressed

UCP-1 and PGC-1α that are highly expressed in brown adipose tissue (Singh et al., 2009b). The mice exhibited an improvement of insulin sensitivity in peripheral tissues. The beneficial outcome of insulin sensitivity with adipose *Atg7* knockout in mice might be due to the higher content of brown adipocytes in adipose tissue.

Host defense and immunity

The autophagy pathway degrades not only endogenous components in the cell but also exogeneous pathogens, such as viruses, parasites and bacteria. The autophagy network is implicated in several pathways targeting the pathogens to the lysosome (Levine et al., 2011). Pathogens that enter host cells by phagocytosis are surrounded by a vesicular structure called phagosome. The fusion of phagosomes with lysosomes or autophagosomes is dependent on autophagy proteins such as Atg5 and Atg7, and the envelopment of phagosomes involves autophagosomal membranes (Lee et al., 2010a; Sanjuan et al., 2007). These processes contribute to the presentation of major histocompatibility complex class II (MHC-II) antigen. In dendritic cells, autophagy is required for the transport of viral nucleic acids to toll-like receptor-7 (TLR7) in endosomes and for the production of interferon-α (Lee et al., 2007). The mechanism by which autophagy regulates cytokine secretion is not clear. Mutations in *Atg16L1* and *Atg5* have been found to disrupt granule exocytosis in intestinal epithelial cells (Cadwell et al., 2008), and this might explain at least in part the role of autophagy in cytokine secretion.

Aging and longevity

Autophagy interacts with several longevity pathways in *Caenorhabditis elegans*, including insulin-like growth factor signaling, TOR signaling, and calorie restriction. These pathways require the activity of autophagy in their effect of lifespan extending (reviewed in (Jia and Levine, 2010)). Mouse models with impaired autophagy have shown accelerated aging (Komatsu et al., 2005), suggesting that a reduced level of autophagy might facilitate aging. This role of autophagy might explain why caloric

restriction, which improves autophagy, slows down the aging process (Bergamini, 2005). One of the major contributions of autophagy might be the protection of DNA damages resulting from cellular stress. Autophagy-defective cells accumulate damaged checkpoint proteins or repair proteins without efficient turnover. Due to suppressed autophagic recycling of cellular contents, these cells may lack sufficient energy for proper DNA replication and repair (Jin and White, 2007; Mathew et al., 2007). Although autophagy promotes cell survival by dealing with stress, an excess level of autophagy could result in cell death (Maiuri et al., 2007). *Drosophila* fat body that over-expresses Atg1 showed reduction in cell size and ultimately resulted in cell loss (Martin and Baehrecke, 2004). Catalase is the major enzymatic scavenger for reactive oxygen species (ROS) that can cause cellular oxidative stress and facilitate aging process. Caspase inhibition induces the selective autophagic degradation of catalases, and causes abnormal ROS accumulation that leads to necrotic cell death. (Yu et al., 2006). Thus, over-activated autophagy could cause a harmful effect and facilitate aging process.

b. Roles of autophagy in human diseases

Although autophagy may be dispensable in rapidly proliferating cells, its housekeeping and renewing function must be important for non-dividing differentiated or senescent cells, such as cardiac- and skeletal-muscle cells, hepatocytes, and neurons. Given the diverse involvement of autophagy in physiological processes, it is not surprising to see that too much or too little of autophagy level could lead to many diseases, which include cardiac- and skeletal-muscle diseases, liver diseases, infectious diseases, neurodegeneration and cancer.

Neurodegeneration

Autophagosomes accumulate in the neurons of patients with neurodegenerative diseases, such as Alzeheimer's disease, Parkinson's disease, and Hungtington's disease, indicating the abnormal level of autophagy in these neurons (Hara et al., 2006; Komatsu

et al., 2006). Autophagy participates in the clearance of aggregate-prone mutant proteins, such as polyglutamine tract-containing proteins in Hungtington's disease, mutant alpha-synucleins in familial Parkinson's disease, and mutated tau proteins in frontotemporal dementia (Martinez-Vicente and Cuervo, 2007; Rubinsztein, 2006; Rubinsztein et al., 2005). Oligomeric and aggregated proteins tend to have a higher toxicity, and because they have difficulty in unfolding and passing through the narrow pore of proteasomal barrel, they are better substrates for autophagic degradation. It needs to be clarified further whether an autophagy defect is a cause of the neurodegenerative diseases or the higher level of accumulated autophagosomes is simply a consequence of the increased protein aggregates. The expression of Beclin 1 is reduced by 60 to 70 % in Alzheimer's disease (Jaeger and Wyss-Coray, 2010). In Huntington's disease, mutations in the dynein motor machinery that impair the autophagic clearance of aggregate-prone proteins were identified (Ravikumar et al., 2005). Autophagy-stimulatory agents such as rapamycin analogs and lithium choloride have a high promise as chemotherapeutics to prevent neurodegeneration (Rubinsztein et al., 2007).

Liver disease

The participation of autophagy in the liver functions is manifested by both genetic disorders and genetic manipulation studies. Alpha1-antitrypsin deficiency is a common genetic liver disease that occurs as a consequence of a point mutation in the *alpha1-antitrypsin Z* (*ATZ*) gene (Stoller and Aboussouan, 2005). The ATZ protein is usually secreted into the blood and body fluids and functions as an inhibitor of neutrophil proteases, while the mutation leads to retaining of the protein in the ER of hepatocytes. Autophagy is activated by ER accumulation of ATZ and the disposal of the mutant ATZ protein is largely dependent on autophagy (Perlmutter, 2006). Atg5-deficient cells showed a reduction in the degradation of the mutant ATZ protein (Kamimoto et al., 2006), indicating the important role of autophagy in the clearance of this protein. Autophagy is also important in the development of alcoholic liver disease (ALD). Sustained over-consumption of alcohol results in accumulation of oxidative stress, lipid droplets and

damaged mitochondria in hepatocytes, which is the base of ALD (O'Shea et al., 2010). Autophagy has a protective effect against the impairment of alcohol in the liver. Ethanol-induced ROS production activates autophagy, which selectively removes damaged mitochondria and lipid droplets in hepatocytes (Ding et al., 2011). Mice treated with rapamycin to increase autopahgy showed reduced hepatotoxicity after acute ethanol exposure, and this potential protective role of autophagy against alcoholic liver injury has led to attempts of therapeutic approaches to treat ALD by autophagy modulation (Ding et al., 2010).

Skeletal- and cardiac-muscle disease

Defective autophagy contributes to inherited skeletal- and cardiac-muscle diseases. Danon disease, characterized by cardiomyopathy, myopathy, and mental retardation, is caused by a mutation in the lysosomal-associated membrane protein-2, which is associated with excessive autophagosomal accumulation (Tanaka et al., 2000). Pompe disease, another skeletal- and cardiac-muscle disorder, results from deficiency of acid alpha-glucosidase, a glycogen-degrading lysosomal enzyme (Fukuda et al., 2006). The defective lysosomal function provokes abnormal protein trafficking and accumulation of autophagosomes.

Autophagy also represents an important response to cardiac stresses, such as ischemia or pressure overload. Patients with coronary artery disease, hypertension, aortic valve disease, and congestive heart failure were found to have an accumulation of autophagosomes in their cardiac biopsy tissues (Terman and Brunk, 2005). Therefore, autophagy may play a dual role in cardio-protection. Excessive autophagy causes damage in myocytes, while in sudden cardiac event autophagy is necessary. The current consensus is that the cytoprotective effects of autophagy outweigh its adverse effect in myocyte degeneration, so promotion of autophagy is being considered as a potential therapeutic strategy in acute cardiac stress conditions (Przyklenk et al., 2012).

Cancer

The *Beclin 1* gene was identified as a haploid insufficient tumor suppressor gene (Liang et al., 1999). The gene was mapped to a tumor susceptibility locus about 150 kb centromeric to the breast cancer *BRCA1*on human chromosome 17q21. Monoallelic loss of chromosome 17q21 is prevalent in human prostate, breast and ovarian cancer (Li et al., 2010). The *Beclin 1*+/− mice showed increased incidence of spontaneous tumors in addition to reduced autophagy activity (Qu et al., 2003). Bcl-2 interacts with Beclin 1 and it functions as an oncogene by blocking apoptotic cell death. Bcl-2 also negatively regulates Beclin 1-dependent autophagy and autophagic cell death (Pattingre and Levine, 2006). Other autophagy proteins that function as tumor suppressors include Bif-1, death-associated protein kinase 1 (DAPK1), liver kinase B1 (LKB1), phosphatase and tensin homolog (PTEN), and UVRAG. Activation of the class I PI3K-Akt-mammalian target of rapamycin (mTOR) signaling pathway suppresses autophagy (Wu et al., 2009), and mutations in *PIK3CA* and *Akt* and amplification/copy gain of *PIK3CB* and *Akt* are found in many cancers (Ogita and Lorusso, 2011; Xing, 2010). The tumor suppressor p53 also regulates autophagy but its effect could be positive or negative depending on its sub-cellular localization (Morselli et al., 2009).

The relation between autophagy and cancer is paradoxical. At the initiation stage of some cancer, autophagy has a negative effect for tumor growth (Degenhardt et al., 2006). The damaged cellular content in tumor-prone cells can be removed by autophagy, which may alleviate the cellular potential to develop as tumor. Autophagy exerts anti-tumor effects in such circumstances. On the other hand, the external environment for tumor cells is often short of nutrients, growth factors, and oxygen due to restricted blood supply. Autophagy can promote tumor cell survival in such an environment that is remote from blood and nutrient supply (Morselli et al., 2009). In developed tumor cells, the regeneration of amino acids through autophagy may provide metabolic substrates and energy for prolonged proliferation (Degenhardt, Mathew et al. 2006). Autophagy is often induced in cancer cells under chemo- or radio-therapy, contributing to the resistance of

cells to the anti-tumor therapies (Morselli et al., 2009). Inhibition of autophagy is therefore considered as a potential useful strategy in combination with the cancer therapies (White and DiPaola, 2009). Most of these trials combine cytotoxic chemotherapy with hydroxychloroquine, which inhibits the terminal lysosome degradation step to block autophagy flux (Chen and Karantza, 2011).

Other diseases

In Paneth cells of the intesine, autophagy plays a protective role against the damage from inflammation thereby suppressing the development of Crohn's disease (Cadwell et al., 2008). Autophagy is also found to act against the deleterious consequences of obesity (Yang et al., 2010). Although the mechanism for this protection is not clear, a clinical application of autophagy stimulation is under consideration for obesity-related disease prevention (Tanaka et al., 2012). Autophagy was reported to facilitate the progression of acquired immune deficiency syndrome (AIDS) (Espert et al., 2007). Human immunodeficiency virus-infected T cells expressing envelope glycoproteins (Env) induce a higher activity of autophagy in uninfected CD4+ T cells that is triggered by the contact of Env with chemokine receptor type 4 (Espert et al., 2007). The Env-mediated autophagy leads to T cell apoptosis, which is commonly found in patient with AIDS (Espert and Biard-Piechaczyk, 2009).

In summary, autophagy is required in major physiological processes and is implicated a number of prevalent diseases, implying that the regulation of autophagy is essential for the health of human body.

III. The ULK1 complex, the key element for autophagy initiation

Despite the well-appreciated functions of autophagy in physiology and diseases, the mechanism of autophagy induction remains elusive. My thesis work has intended to

better understand the molecular mechanisms of the early steps of autophagy induction with a particular focus on the ULK1 protein complex. This section will introduce the ULK1 complex in more detail, and discuss the functions and the regulatory mechanisms of the ULK1 complex.

a. The Atg1/ULK1 protein kinase

A cornerstone for our understanding of autophagy is a genetic screen in *Saccharomyces Cerevisiae* that discovered a set of autophagy-related genes including Atg1, an evolutionarily conserved Ser/Thr kinase that functions at the most upstream step of autophagy induction (Matsuura et al., 1997). In *C. elegans*, the *Atg1* homologue is *uncoordinated-51 (Unc-51*), which was shown to affect starvation-dependent formation of dauer (the stage that the larva goes into stasis and survives harsh conditions) (Melendez et al., 2003). Interestingly, *Unc-51* was originally discovered in a screen for uncoordinated movement in *C. elegans* (Kuroyanagi et al., 1998; Yan et al., 1998), and that is where the name comes from. It was found later that the unc-51 protein participates in axon guidance (Lai and Garriga, 2004) and neuronal vesicular movement (Sakamoto et al., 2005), which explains its role in movement coordination. *Drosophila Melanogaster* has one *Atg1* homolog identified so far, and deletion of the *Drosophila Atg1* gene results in a defective autophagy phenotype (Scott et al., 2004). Similar to *Unc-51* in *C. elegans*, the *Drosophila Atg1* is also crucial in neuronal development (Toda et al., 2008), possibly through interacting with cytoskeleton motor proteins to direct synaptic vesicle transport (Chen et al., 2008; Toda et al., 2008). The shared funcrion of *Atg1* and *Unc-51* in neuronal vesicular trafficking and autophagy implies an important role of cytoskeleton in both processes.

While yeast, *C. elegans* and *Drosophila* have only one homolog for *Atg1* (Kamada et al., 2000; Matsuura et al., 1997), mammals have five members of Atg1

homologues that are named *ULK1, ULK2, ULK3, ULK4,* and *serine/threonine kinase-36 (STK36)* (Figure 3). Among them, STK36 is remotely related to the *Drosophila* Fused protein. All the five ULK family kinases have a highly conserved N-terminal kinase domain (Chan et al., 2007; Hara et al., 2008; Jung et al., 2009; Kundu et al., 2008; Yan et al., 1998; Yan et al., 1999; Young et al., 2009). ULK1 and ULK2, which were shown to regulate autophagy (Chan et al., 2007; Chan et al., 2009; Jung et al., 2009), contain a proline/serine-rich region in the middle. The two kinases also contain a C-terminal region involved in interaction with other autophagy proteins such as Atg13 (Yan et al., 1999). In contrast to the neonatal lethality when other major autophagy genes (*Atg3, Atg5, Atg7, Atg9*) are knocked out in mice (Komatsu et al., 2005; Kuma et al., 2004; Saitoh et al., 2009; Sou et al., 2008), *ULK1* knock-out mice are viable (Kundu et al., 2008). This appears to be due to the complementation by ULK2. A recent study has shown that overexpression of ULK3 activated autophagy in IMR-90 cell lines (Young et al., 2009). Moreover, upon autophagy induction by amino acid or serum depletion, ULK3 co-localized with the Atg12-Atg5 conjugate in a punctuate pattern (Young et al., 2009). Thus, ULK3 appears to be involved in a specific step of autophagy and its function is different from that of ULK1 and ULK2. It remains unknown whether ULK4 and STK36 regulate autophagy.

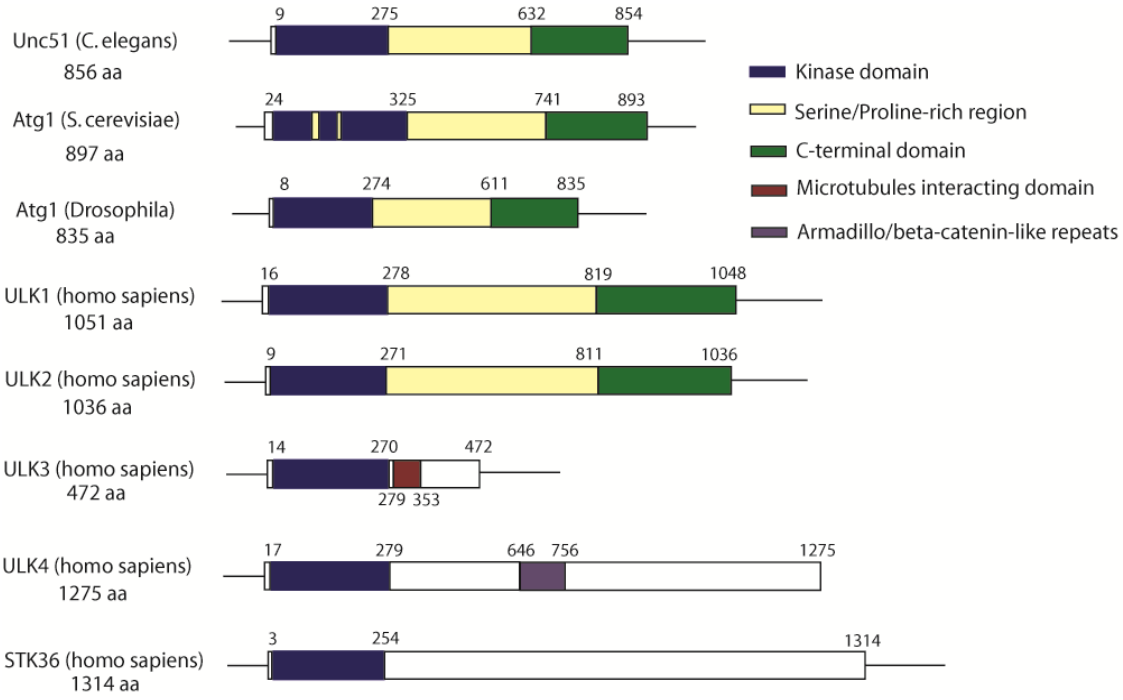


Figure 3. Sequence comparison of Atg1 homologues in different species.

Domain structures of *Caenorhabditis elegans* Unc51, *Saccharomyces cerevisiae* Atg1, *Drosophila* Atg1, *homo sapiens* ULK1, ULK2, ULK3 , ULK4, and STK36 amino acid sequences (Jung et al., 2010). Sequence of amino acid encoding the starting and ending residues for each domain is indicated.

b. The Atg1/ULK complexes

In yeast, Atg1 interacts with Atg13, Atg17, Atg29 and Atg31 (Funakoshi et al., 1997; Kabeya et al., 2009). The interaction of Atg1 with Atg13 and Atg17 is regulated in a manner dependent upon the kinase activity of Atg1 and TOR (target of rapamycin), whereas the interaction of Atg17 with Atg29 and Atg31 appear to be consistent (Cheong et al., 2005; Kabeya et al., 2009). In *Drosophila*, Atg1 binds to Atg13 but it has not been determined whether Atg1 has other binding partners (Chang and Neufeld, 2009). In contrast to the interaction between yeast Atg1 and Atg13, the interaction between *Drosophila* Atg1 and Atg13 is not regulated by TOR activity (Chang and Neufeld, 2009). *Drosophila* Atg13 appears to function positively and negatively on Atg1 and autophagy (Chang and Neufeld, 2009). In *C. elegans*, Unc-51 directly interacts with epg-1 that is a homolog of Atg13 (Tian et al., 2009). Knockout of epg-1 inhibited autophagy, suggesting that the Unc51-epg1 complex might have the conserved function in autophagy (Tian et al., 2009).

In mammalian cells, ULK1 and ULK2 interact with multiple proteins including mammalian Atg13. The mammalian Atg13 constitutively interacts with both ULK1 or ULK2 and plays crucial roles in autophagy induction (Ganley et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009). ULK1 and ULK2 also interact with FIP200 (Focal adhesion kinase family interacting protein of 200 kDa) (Hara et al., 2008)(Ganley, Lam du et al., 2009; Hosokawa, Hara et al., 2009; Jung, Jun et al., 2009. FIP200 has been postulated to be a functional homologue of yeast Atg17 in mammalian cells (Hara et al., 2008), although their sequence similarity is low. FIP200 is important for the localization of ULK1 to the isolation membranes (Hara et al., 2008). Atg13 and FIP200 do not contain any enzymatic domains and likely function as scaffolding proteins in recruiting substrates of ULK (Chan et al., 2009; Ganley et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009). Atg101, which does not show any homology with known autophagy

proteins, interacts with ULK1 and Atg13 and regulates autophagy induction (Mercer et al., 2009). These ULK binding proteins are potential substrates of ULK1 (Jung et al., 2009), while their functions remain largely unknown.

Recent studies have shown that ULK1 can interact with the Hsp90-Cdc37 chaperone complex (Behrends et al., 2010; Joo et al., 2011). ULK1 was also found to interact with ubiquitin-specific protease 10 (Sowa et al., 2009), implying a potential link between the lysosome-dependent autophagic degradation pathway and the proteasome-dependent degradation pathway. ULK1 also interacts with mammalian target of rapamycin complex 1 (mTORC1) (Chan et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009) and 5' AMP-activated protein kinase (AMPK) (Egan et al., 2011; Kim et al., 2011; Shang et al., 2011). mTOR and AMPK are key nutrient- and energy-sensing kinases (Hardie et al., 2012; Laplante and Sabatini, 2012). These two kinases phosphorylate and regulate ULK1 (Egan et al., 2011; Kim et al., 2011; Shang et al., 2011). The regulation of ULK1 by mTORC1 and AMPK will be discussed in the following section in detail.

c. Regulation of the Atg1 complex in lower eukaryotes

TOR is a key negative regulator of autophagy induction (Kamada et al., 2004). As an evolutionarily conserved serine/threonine protein kinase that belongs to the phosphatidylinositol kinase-related kinase family, TOR is the major nutrient-sensing protein kinase in eukaryotes and its activity is enhanced by high nutritional and energy status (Sengupta et al., 2010). Through sensing the cellular nutritional status, TOR regulates cellular growth and autophagy. Nutrient starvation or specific TOR inhibitors like rapamycin can induce autophagy, and the induction was found to depend on the active state of Atg1 (Kamada et al., 2000; Matsuura et al., 1997). These studies have suggested that Atg1 mediates the TOR inhibitory signaling to the autophagy induction machinery. The relation between TOR and Atg1 has been verified in several lower

eukaryotes such as *S. cerevisiae, Dictyostelium discoideum, C. elegans* and *Drosophila* (Melendez et al., 2003; Otto et al., 2004; Scott et al., 2007).

In yeast, TOR inhibits autophagy by destabilizing the association between Atg1 and its binding partners (Funakoshi et al., 1997; Kabeya et al., 2005; Kabeya et al., 2009; Kamada et al., 2000; Kawamata et al., 2008). Under nutrient-enriched conditions, TOR phosphorylates Atg13 at multiple sites (Kamada et al., 2010). As a consequence, the phosphorylation induces dissociation of Atg13 from Atg1, thus suppressing autophagy induction (Funakoshi et al., 1997; Kabeya et al., 2005; Kamada et al., 2000). When TOR is inhibited under starvation conditions, Atg13 is no longer phosphorylated by TOR. Atg1 is then able to interact with Atg13, and the kinase activiy of Atg1 is enhanced (Kamada et al., 2009) (Figure 4).

The yeast protein kinase A (PKA) was found to negatively regulate autophagy because activation of PKA activity inhibited autophagy and inactivation of the PKA pathway induced autophagy (Stephan et al., 2010). The regulation of PKA on autophagy was thought to be mediated through the Atg1 complex. PKA directly phosphorylates Atg1 (Budovskaya et al., 2005) and Atg13 (Stephan et al., 2010). The phosphorylation by PKA promotes Atg13 localization to the pre-autophagosome (PAS, the membrane origination of autophagosome in yeast), and the phosphorylation sites are distinct from the TOR phosphorylation sites, indicating PKA and TOR function independently to regulate autophagy through the Atg1/Atg13 complex. The yeast AMPK homolog sucrose non-fermenting 1 (Snf1) can positively regulate autophagy (Wang et al., 2001b), but the exact mechanism is not understood. Snf1 is regulated by Elm1, Sak1, Tos3, and ROS (Hedbacker and Carlson, 2008) as well as by TORC1 (Orlova et al., 2006).

In contrast to the TOR-dependent interaction between Atg1 and Atg13 in yeast, the Atg1-Atg13 interaction in *Drosophila* is independent of TOR (Chang and Neufeld, 2010). (Figure 5). The nutritional status regulates the PI3K-Akt-TOR pathway and as a consequence, regulates autophagy induction. This regulation is in a manner dependent

upon Atg1 (Dutta and Baehrecke, 2008; Scott et al., 2007). Under nutrient-enriched conditions, the active *Drosophila* TOR hyperphosphorylates Atg1 and phosphorylates Atg13 to suppresses autophagy. Atg1 also phosphorylates itself. Under starvation conditions, the inactive TOR no longer phosphorylates Atg1 and Atg13. Atg1 still autophosphorylate and it phosphorylates Atg13 to induce autophagy (Chang and Neufeld, 2010).

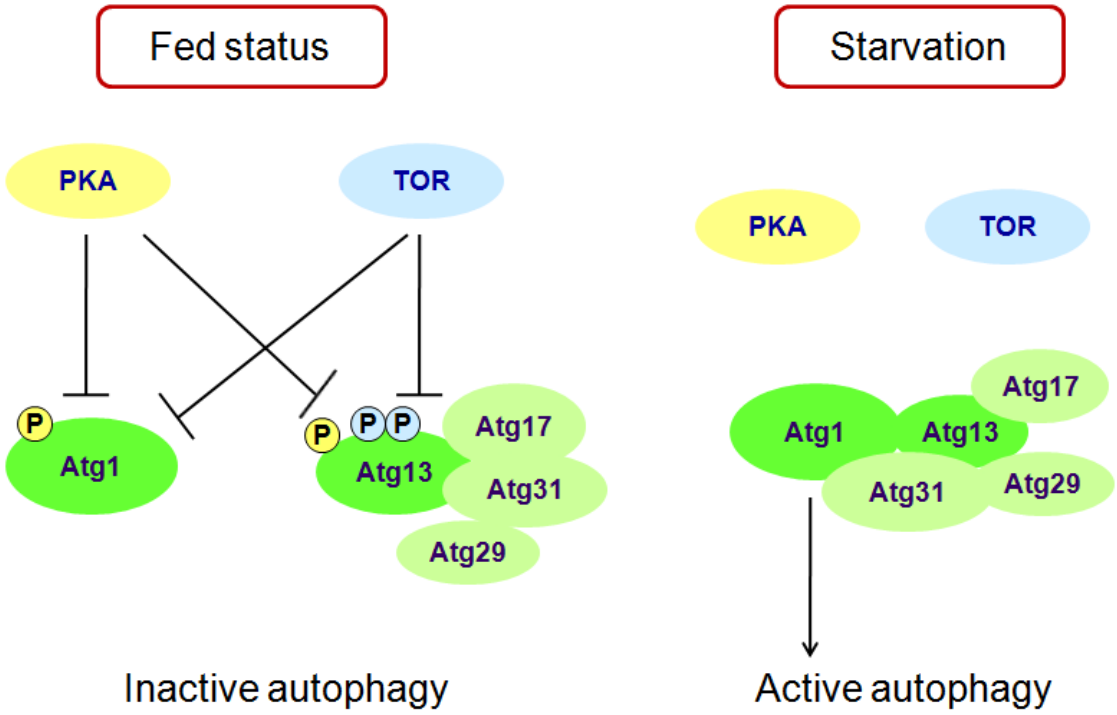


Figure 4. Regulation of the Atg1 complex in *Saccharomyces cerevisiae*.

The yeast Atg1 complex consists of Atg1, Atg13, Atg17, Atg29 and Atg31 (Funakoshi et al., 1997; Kabeya et al., 2009). Under nutrient rich conditions, TOR phosphorylates Atg13 and induces dissociation of Atg13 from Atg1 and its other binding partners (Atg17, Atg29 and Atg31) (Funakoshi et al., 1997; Kabeya et al., 2005; Kamada et al., 2000). When TOR is inhibited in starvation conditions, Atg13 is no longer phosphorylated by TORC1 and stably interacts with other proteins in the complex. In this state, the kinase activiy of Atg1 is increased and autophagy is induced (Kamada et al., 2009). The yeast PKA negatively regulates autophagy (Stephan et al., 2010) by phosphorylating Atg1 (Budovskaya et al., 2005) and Atg13 (Stephan et al., 2010). The "P" sign for phosphorylation is shown in the same color as the kinase responsible for the phosphorylation.

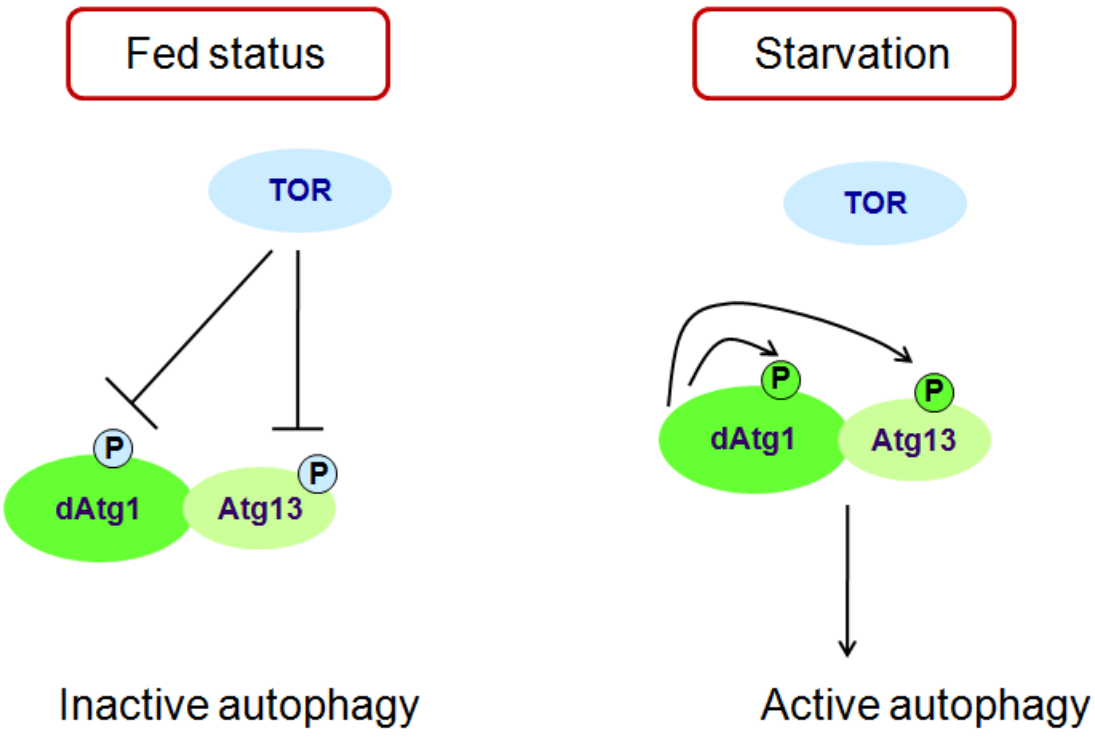


Figure 5. Regulation of the dAtg1 complex in *Drosophila*.

The *Drosophila* Atg1 (dAtg1) interacts with Atg13 independently of TOR activity. Active TOR phosphorylates Atg13 and hyperphosphorylates dAtg1 to suppresses autophagy. dAtg1 autophorylates. When TOR is inactive, dAtg1 still phosphorylate itself and it also phosphorylates Atg13 to induce autophagy. The "P" sign for phosphorylation is shown in the same color as the kinase responsible for the phosphorylation.

d. Regulation of the ULK1 complex in mammalian cells

The regulatory mechanism of Atg1 in lower eukaryotes is partially conserved in higher eukaryotes. mTOR, like yeast TOR, plays a key role in sensing nutritional and energy status of cells to regulate cell growth and autophagy. The function of mTOR is implicated in many pathological conditions such as cancer, aging, longevity, metabolic disorders, and neurodegenerative diseases (Cota, 2009; Garelick and Kennedy, 2010; Kapahi et al., 2010; Liu et al., 2009a; Sabatini, 2006). mTOR forms two distinct protein complexes. The complex containing raptor (regulatory-associated protein of mTOR) is called mTORC1 (mTOR complex 1), while the complex containing rictor (raptor-independent companion of mTOR) is called mTORC2 (Guertin and Sabatini, 2007; Jacinto et al., 2006; Kim et al., 2002; Sarbassov et al., 2004). mTORC1 is responsible for sensing cellular nutrient and energy status and for the regulation of cell growth and autophagy (Jung et al., 2010; Sabatini, 2006).

mTOR responds not only to the cellular availability of specific nutrients and stress but also to circulating growth factors such as insulin which corresponds to the nutritional status of a multicellular organism. Insulin or insulin-like growth factors activates the PI3K-Akt pathway. As a consequence, Akt phosphorylates TSC2 (tuberous sclerosis complex 2) and thereby inhibiting the GTPase activating function of TSC2 toward a small GTPase named Rheb (Inoki et al., 2003a; Inoki et al., 2002; Potter et al., 2002; Zhang et al., 2003). Rheb is an activator of mTORC1, thus the inhibition of TSC2 by Akt results in activation of mTORC1. Akt can also affect mTORC1 activity through phosphorylation of the proline-rich Akt substrate (PRAS40) (Sancak et al., 2007; Vander Haar et al., 2007), a component of mTORC1 (Kovacina et al., 2003). mTORC1 can also be activated by other growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) via mitogen-activated protein kinase (MAPK) activation (Carriere et al., 2008).

In nutrient-enriched conditions, mTOR interacts with ULK1 and phosphorylates ULK1 at Ser758 (Ser757 in mouse ULK1) (Kim et al., 2011; Shang et al., 2011) and Ser658 (Shang et al., 2011) (Figure 6). These phosphorylations strengthen the interaction between mTOR and ULK1 and inhibit ULK1's kinase activity (Chan et al., 2009; Ganley et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009). Unlike the interaction between Atg1 and Atg13 in yeast, the interaction between ULK1 and Atg13 in mammalian cells is not regulated by mTORC1 (Chan et al., 2009; Ganley et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009). Under starvation or rapamycin-treated conditions, mTORC1 no longer phosphorylates ULK1 (Jung et al., 2009) (Ganley et al., 2009; Hosokawa et al., 2009a). In this state, ULK1 appears to be more active in its kinase activity. S318 in Atg13 has been identified as a phosphorylation site by ULK1 (Joo et al., 2011). The phosphorylation was found to be important for Parkin-induced mitophagy.

Another key regulator of ULK1 is AMPK, a serine/threonine protein kinase ubiquitiously expressed and highly conserved in eukaryotes. AMPK exists as a protein complex containing three subunits named alpha, beta and gamma (reviewed in (Hardie et al., 2012)). Calcium calmodulin-dependent protein kinase kinase (CaMKK) and tumor suppressor STK11/LKB1 regulate the activity of AMPK. They phosphorylate Thr172 of AMPK alpha catalytic subunit and activate AMPK (Hawley et al., 2003; Hong et al., 2003; Woods et al., 2003). CaMKK and LKB1 are activated by intracellular calcium levels and the AMP:ATP ratio (Hong et al., 2003). A recent study showed that mitochondrial uptake of Insp3R-released calcium is fundamental for oxidative phosphorylation and ATP production (Cardenas et al., 2010). Reduction of this calcium uptake results in reduced ATP production and activation of AMPK. The AMPK activity is also regulated by circulating hormones and cytokines, including bradykinin and the adipokines leptin and adiponectin through unknown mechanism (Xu et al., 2012). Pharmaceutical reagents like the anti-diabetic metformin, thiazolidinediones and peroxisome proliferator-activated receptor alpha (PPAR-α) agonist fenofibrate also activate AMPK through unknown mechanism (Gruzman et al., 2009).

AMPK can indirectly induce autophagy through inhibiting mTORC1. AMPK phosphorylates and activates TSC2, the inhibitor of mTORC1, thus resulting in suppression of mTORC1 (Inoki et al., 2003b). AMPK also phosphorylates raptor at Ser722 and Ser792 resulting in inhibition of mTORC1 (Gwinn et al., 2008).

AMPK also directly phosphorylate ULK1 to induce autophagy (Egan et al., 2011; Kim et al., 2011; Shang et al., 2011). The phosphorylation was shown to be required for the activation of ULK1 in macroautophagy and mitophagy induction (Egan et al., 2011; Kim et al., 2011). The association of AMPK with ULK1 results in multiple phosphorylations of ULK1 by AMPK on Ser317, Ser467, Ser555, Thr575, Ser638 (Ser637 in mouse) and Ser777 (Egan et al., 2011; Kim et al., 2011). Dephosphorylation of ULK1 by mTOR at Ser758 and phosphorylations by AMPK on S317, S555, and S777 appear to be important for the activation of ULK1 for autophagy induction. The binding and phosphorylation of ULK1 by AMPK is prevented by mTORC1-mediated phosphorylation of ULK1 (Egan et al., 2011; Kim et al., 2011). This way mTOR and AMPK counteract to control autophagy induction (Figure 6). mTOR regulates ULK2 in a similar pattern as ULK1 (Chan et al., 2009; Hosokawa et al., 2009a; Jung et al., 2009) but the regulation of ULK2 by AMPK remains to be determined.

Like yeast PKA, mammalian PKA regulates autophagy via Atg1/ULK1. However, the regulator mode is different. In mammalian cells, PKA activates mTORC1 (Mavrakis et al., 2006) and it appears to involve phosphorylation of PRAS40 by PKA (Blancquaert et al., 2010) or phosphorylation and inactivation of AMPK by PKA (Djouder et al., 2010).

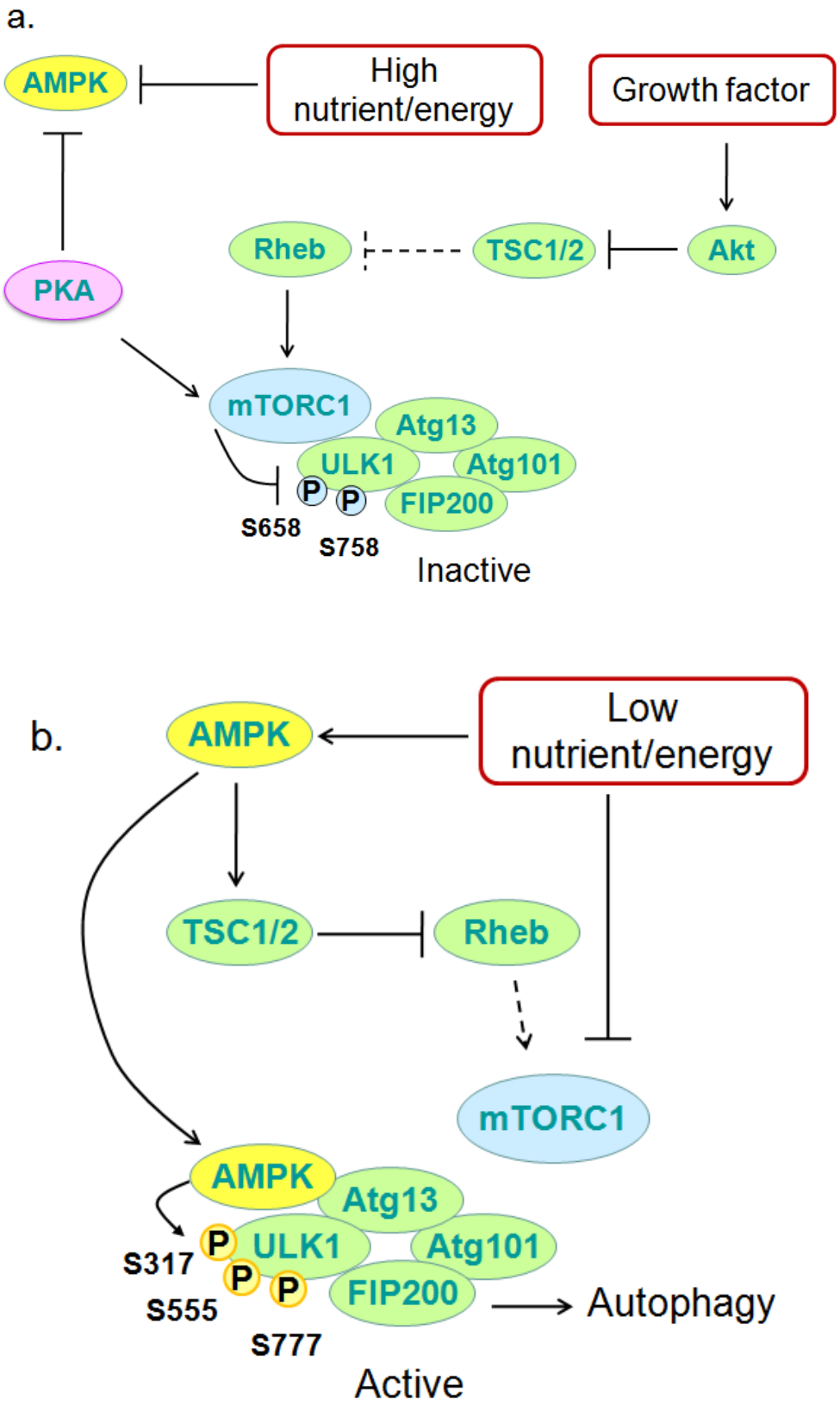

a.
AMPK
High nutrient/energy
Growth factor
Rheb
TSC1/2
Akt
PKA
mTORC1
Atg13
ULK1
Atg101
P
P
FIP200
S658
S758
Inactive
b.
AMPK
Low nutrient/energy
TSC1/2
Rheb
mTORC1
AMPK
Atg13
P
ULK1
Atg101
S317
P
P
FIP200
Autophagy
S555
S777
Active

Figure 6. Regulation of ULK1 in mammals.

(a) The ULK1 complex is inactive under nutrient-enriched conditions. AMPK does not interact with ULK1 and mTOR phosphorylation of ULK1 inhibits the ULK1 complex (Hosokawa et al., 2009a; Jung et al., 2009). PKA activates TORC1 (Mavrakis et al., 2006) and it can also phosphorylate and inactivate AMPK (Djouder et al., 2010).
(b) The ULK1 complex is active under nutrient-starved conditions. Inactive mTOR disassociates from the ULK1 complex. AMPK binds to ULK1 and phosphorylates ULK1 to activate it (Egan et al., 2011; Kim et al., 2011). The "P" sign for phosphorylation is shown in the same color as the kinase responsible for the phosphorylation. Arrows pointing to the "P" sign indicate activating phosphorylation, and blunted lines pointing to the "P" sign indicate inhibiting phosphorylation.

e. How the ULK1 complex regulates autophagy.

The ULK1 complex was originally found to function at the initial stage of autophagy, but it has also been shown to regulate other stages of autophagy (Figure 5). Under nutrient rich conditions, ULK1 is located in dispersed area in the cytoplasm, while in conditions favoring autophagy induction ULK1 is re-distributed to form puncta on the ER (Itakura and Mizushima, 2010). The ULK1 puncta colocalizes with Atg14L, Atg16L and WIPI-1, and localizes adjacently to DFCP1 (Itakura and Mizushima, 2010). These proteins localize to the isolation membrane or elongating isolation membranes (Fujita et al., 2008; Matsunaga et al., 2010; Polson et al., 2010), suggesting that ULK1 is localized to the isolation membrane. Kinase-dead forms of ULK1 and ULK2 are able to localize to the isolation membrane in mammalian cells (Matsunaga et al., 2010; Matsunaga et al., 2009). This suggests that the localization of ULK1/2 to the isolation membrane does not depend on the kinase activity of ULK1/2. The formation of the isolation membrane was suppressed in FIP200-deficient MEFs (Itakura and Mizushima, 2010), suggesting that the integrity of the ULK1 complex is required for the formation of the isolation membrane.

Another key component involved in the formation of isolation membrane is the Atg14L-containing PI3KC3 complex that is responsible for the production of PI3P on the ER membrane (Matsunaga et al., 2009; Zhong et al., 2009). The PI3KC3 complex recruits DFCP1 and WIPI family proteins to the site where the isolation membrane formation is initiated (Axe et al., 2008; Matsunaga et al., 2010; Polson et al., 2010). In *Drosophila*, Atg1 regulates PI3P localization to autophagosome upon starvation (Juhasz et al., 2008). Similarly, in mammalian cells ULK1 is required for the formation of Atg14L puncta (Itakura and Mizushima, 2010). ULK1 puncta could still be formed in cells treated with wortmannin, the inhibitor of PI3KC3, suggesting that the ULK1 complex might function upstream of the PI3KC3 complex (Itakura and Mizushima, 2010). Although these studies have supported the stimulatory function of ULK1 on Atg14L-PIKC3 complex, it has not been directly demonstrated that ULK1 regulates the activation of Atg14-containing PI3KC3 complex. A recent finding of the Exocyst

complex in the autophagy pathway introduces another tilt in the mechanism of Atg14L-PI3KC3 complex activation with respect to the function of ULK1 in the activation of PI3KC3 and autophagy induction (Bodemann et al., 2011). In this study, a Ras-like small G protein, RalB, was found to interact with its effector Exo84 in the Exocyst complex. Through the interaction, RalB induces the assembly of ULK1 complex and Atg14L-Beclin 1-PI3KC3 complex on the Exocyst (Bodemann et al., 2011).

During the elongation and maturation steps of the autophagosome formation in yeast, Vps34 plays an important role in the recruitment of Atg5-Atg12 conjugation complex to PAS in yeast (Suzuki et al., 2001). Although this finding supports the possibility that Atg1/ULK1 functions upstream of Atg5, the localization of ULK1/2 to the isolation membrane has been shown to depend on Atg5 (Hara et al., 2008). Thus, it appears that ULK1/2 and Atg5 are inter-dependent during the formation of the isolation membrane. A recent study, however, provides a different view on the inter-relationship between ULK1/2 and Atg5 by providing evidence that autophagy can be induced independently of Atg5/Atg7 but dependently of ULK1 (Nishida et al., 2009). This implies that there might exist multiple types of autophagy in mammalian cells. The PE-conjugated Atg8 homologues, such as LC3, GATE16 and GABARAP, are important for the autophagic membrane elongation (Weidberg et al., 2010). The yeast Atg1 interacts with Atg8 through an Atg8 interacting motif (AIM), through which Atg1 interacts with the isolation membrane (Nakatogawa et al., 2012). This interaction is important for Atg1 localization to the isolation membrane. Similarly, ULK1 interacts with Atg8 proteins such as GATE16 and GABARAP via an LC3-interaction region (LIR) (Kraft et al., 2012; Okazaki et al., 2000) .

ULK1 also plays an important role in the regulation of Atg9 function during the membrane elongation process. Atg9 shuttles between TGN and other membrane compartments in the cytoplasm. This shuttling was proposed to play an important role in recruiting membrane fractions to the isolation membrane formation (Orsi et al., 2012). ULK1 knockdown in HEK293 cells interfered with the proper shuttling of Atg9 (Young

et al., 2006). A recent study has shown that Atg1/ULK1 phosphorylates and activates spaghetti-squash activator (Sqa) in *Drosophila* or zipper-interacting protein kinase (ZIPK, also known as DAPK3) in mammalian cells under starvation (Tang et al., 2010). Sqa and ZIPK belong to the DAPK serine/threonine protein kinase family. The activated DAPK in turn phosphorylates myosin II regulatory light chain to activate myosin, the motor protein moving on the actin filaments and driving the trafficking of Atg9-containing membranes. Through the process, Atg9 trafficking from the TGN to the isolation membrane can be regulated by ULK1 (Tang et al., 2010). Another interesting finding is that mammalian Atg9 has a similar sub-cellular distribution as WIPI-2, an effector of PI3P (Jeffries et al., 2004). WIPI-2 is one of the human counterparts of yeast Atg18. In yeast, Atg9 interacts with Atg18 in a manner dependent upon Atg1 (Reggiori et al., 2004). Thus, Atg1/Ulk1 appears to regulate Atg9 through multiple pathways. However, a recent study has shown that the Atg9 shuttling could happen independently of ULK1 (Orsi et al., 2012). Thus, further studies are necessary to clarify the role of ULK1 in the regulation of Atg9.

f. Non-autophagic functions of ULK1.

Although the major functions of ULK1 and ULK2 are related to autophagy regulation, they were shown to regulate cellular processes that are not directly linked to autophagy. In *C. elegans*, Unc-51 was first characterized for regulating outgrowth of mouse neurons (Tomoda et al., 1999). Loss of Unc-51 function resulted in axonal elongation defects and thus an uncoordinated phenotype (Ogura et al., 1994). (Ogura et al., 1994). Loss of other autophagy genes, such as epg-1 (homolog of Atg13) in *C. elegans*, did not yield this phenotype (Tian et al., 2009). This suggests a unique role of Unc-51 in neuronal development (Ogura and Goshima, 2006; Tian et al., 2009). The role of Unc-51 in axonal elongation may be attributed to its interaction with Unc-14 (Ogura et

al., 1997) and VAB-8L (Lai and Garriga, 2004). In *Drosophila*, Atg1 plays an important role in axonal transport through its binding with Unc-76 (Toda et al., 2008).

ULK1 was shown to interact with paxillin (Chen et al., 2008), SynGAP, syntenin (Tomoda et al., 2004), and TrkA/NGF receptor/p62 (Zhou et al., 2007). The interaction between ULK1 and TrkA/NGF receptor/p62 has been shown to be important for the endocytic processes (Zhou et al., 2007). The binding of ULK1 to SynGAP, syntenin and TrkA/NGF receptor/p62 suggests a role of ULK1 in filopodia extension and axon branching (Tomoda et al., 1999; Tomoda et al., 2004; Zhou et al., 2007). FIP200, as a component of the ULK complex, interacts with stathmin, Pyk2, FAK, p53, TSC1, ASK1, and TRAF2 (Gan and Guan, 2008). Although it is unclear whether these proteins also interact with ULK1 and Atg13, it is possible that ULK1/2 may be involved in processes other than autophagy through FIP200 and its binding proteins.

The Atg1/Ulk1 complex appears to function not only as a downstream effector of mTORC1 but also as an upstream effector of mTORC1. *In Drosophila*, Atg1 was shown to negatively regulate TORC1 signaling (Scott et al., 2007). Both ULK1 and ULK2 were also shown to negatively regulate mTORC1 signaling in mammalian cells (Dunlop et al., 2011; Jung et al., 2011; Lee et al., 2007b). A recent study has shown that ULK1 promoted phosphorylation of raptor at Ser855, Ser859, and Ser792 (Dunlop et al., 2011). ULK1 over-expression increased the phosphorylation of raptor at Ser863 and the phosphorylation of mTOR at Ser2481 (Dunlop et al., 2011). Overexpression of ULK1 inhibited the phosphorylation of mTORC1 substrates, S6K1 and 4E-BP1 and it inhibits the binding of raptor with 4E-BP1 (Dunlop et al., 2011; Jung et al., 2011). These findings suggest that TOR and Atg1/Ulk intimately crosstalk for optimal coordination of the cellular processes involving cell growth and autophagy. ULK1 was also shown to phosphorylate the three subunits of AMPK and (Loffler et al., 2011). Although the functional consequence of the phosphorylations remains unclear, the phosphorylations appear to negatively regulate the activity of AMPK. Thus, ULK1 may induce the termination of the autophagy processes by inhibiting AMPK to prevent an excessive

autophagy. Further studies are necessary to clarify this regulatory mechanism and the physiological significance of this potential regulation.

g. Missing gaps in the regulation of the ULK1 complex.

As summarized above, the ULK1 complex participates in the early steps of the isolation membrane formation. However, the molecular mechanisms remain unclear. In particular, how ULK1 is recruited and activated to the site of the isolation membrane formation in the first place remains poorly understood. My thesis work has aimed to better understand the mechanisms of the Ulk1 complex and led to a discovery of a novel component of the complex , which is named MCF.2 cell line derived transforming sequence-like 2 (MCF2L2). Because this protein has an implicated function in the regulation of actin cytoskeleton, my work has involved characterization of the function of this protein in cytoskeletal regulation. Importantly, the role of actin cytoskeleton in autophagy process remains barely understood. Below, I have briefly introduced the critical information on actin cytoskeletal regulation related to my study and how actin cytoskeleton could play a role in autophagy process.

IV. Cytoskeleton proteins in autophagy

The roles of cytoskeletal proteins in autophagy induction remains poorly understood. There are several studies supportive of the important roles of actin cytoskeletal proteins in the regulation of autophagy induction (Monastyrska et al., 2008; Zhu et al., 2011). One study showed that F-actin was reported to form at the site of autophagosome-lysosome fusion (Lee et al., 2010b), and the formation of F-actin (actin nucleation) is subject to regulation of Rho GTPase guanine nucleotide exchange factor (GEF). In addition, the yeast Atg9 interacts with actin nucleation factor Arp2 during its trafficking between Golgi, endosome and PAS (Monastyrska et al., 2008), and therefore

presents a potential connection between actin nucleation and the autophagy pathway. As mentioned above, Atg1/ULK1 phosphorylates and activates Sqa in *Drosophila* and ZIPK in human, which in turn phosphorylate myosin II regulatory light chain to activate myosin. Myosin is regulated by Rho-GTPase effector—Rho-associated kinases (ROCK), and it acts as a cargo carrier for Atg9 in the membrane cycle. The following section will briefly introduce the current knowledge on Rho GTPases, which is an important player in both actin nucleation and myosin regulation.

a. Rho-GTPases

The family of Rho GTPases controls a wide variety of signal transduction pathways in eukaryotes. The major functions include the regulation of the actin cytoskeleton, cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity (reviewed in (Etienne-Manneville and Hall, 2002)). The GTPases cycle between the two exchangeable states: the GTP-bound active form and the GDP-bound inactive form. The GTP-bound active state of GTPases elicits response in target proteins. The hydrolysis of GTP to GDP inactivates GTPases. Rho GTPase belongs to the Ras GTPase superfamily, which falls into five major groups: Ras, Rho, Rab, Arf and Ran. The human genome contains over 60 activators (GEF) and over 70 inactivators (GAP) for the Rho family GTPases. The Rho-, Rac- and Cdc42-GTPases are the three best-characterized members of the Rho GTPase family, and so far there are over 60 targets identified for these three GTPases (Etienne-Manneville and Hall, 2002). The following sections will elaborate on two pathways that have a possible linkage with the autophagy machinery.

b. Actin nucleation

Actin is one of the most abundant and highly-conserved proteins in eukaryotes. Globular (G)-actin is a 42 kDa monomeric ATP-binding protein, and it can be converted into filamentous (F)-actin through polymerization (also called nucleation). The actin filaments create structural scaffolds and support, and serve as tracks for motor proteins. The first major actin nucleator identified was the Arp2/3 complex, a 220 kDa factor made of seven stably-associated polypeptides which are highly conserved in almost all eukaryoteic organisms. Purified Arp2/3 complex showed the ability to bind to the side of an existing actin filament in *vitro* (Welch et al., 1997). Arp2 and Arp3 dimerize at an angle as the first two subunits of a *de novo* filament. The addition of new actin molecules continues at a Y-branch angle based on the existing filament (Goley and Welch, 2006).

The Arp2/3 complex sits on the pointed end of nascent filament as a cap, allowing the barbed end to elongate freely. ATP binds to Arp2 and the hydrolysis of ATP provides the driving force for both actin nucleation and debranching (Dayel and Mullins, 2004; Goley et al., 2004; Zencheck et al., 2009).

Purified Arp2/3 complex only contains very little catalyzing efficiency, and its binding to the actin filaments enhances the activity of Arp2/3 (Nolen et al., 2004). Several more factors are required for potent actin polymerization including phosphorylation of threonine and tyrosine residues in Arp2 (LeClaire et al., 2008), and more critically, the engagement of WCA domain-containing proteins encoded by microbial pathogens or the mammalian nucleation-promoting factors (NPFs) complex (Goley and Welch, 2006).

The WCA domain is comprised of one or several WASP-homology (WH) motifs binding to actin monomers, together with an amphipathic connector region and acidic peptides collectively binding the Arp2/3 complex. Class I mammalian NPFs are a huge family of proteins with WCA domains at the C-terminus and are subcategorized into five

groups based on the N-terminal sequences: (1) the WASP (originally named after the Wiskott-Aldrich Syndrome protein) and N-WASP superfamily, (2) three WAVE (WASP-family verprolin homolog) isoforms, and three more recently identified categories: (3) WASH (WASP/Scar homolog), (4) WHAMM (WASP homolog associated with actin, membranes and microtubules), and (5) JMY (junction-mediating regulatory protein) (Kurisu and Takenawa, 2009; Stradal and Scita, 2006).

Linear actin filament networks enhance the rate of filament formation and solidify the filament organization. The nucleation in these processes is regulated by WASH together with other nucleators (Chhabra and Higgs, 2007; Wang and Riechmann, 2008). Rho1 in the Rho family of small GTPases is a key regulator upstream of WASH in the linear actin filament formation (Goode and Eck, 2007). WASP and WAVE are regulated by the Rho GTPases Cdc42 and Rac, respectively (Liu et al., 2009b), and they are required for the branched actin filament activities (Stradal et al., 2004; Takenawa and Suetsugu, 2007). WH1 domain in WASP and N-WASP is linked to the GTPase binding/Cdc42 and Rac interactive binding domain, which physically interacts with the Rho small GTPase Cdc42 (Pollitt and Insall, 2009).

c. ROCK-myosin pathway

Another mechanism through which Rho GTPases might regulate autophagy involves the regulation of myosin by ROCK. There are two ROCK isoforms: ROCK1 (ROKβ, p160ROCK) and ROCK2 (ROKα). ROCK1 and ROCK2 share 65% homology in the entire amino acid sequence and 92% homology in the kinase domains. The two isoforms are both ubiquitously expressed, while ROCK1 expression is enriched in lung, liver, spleen, kidney, and testis, and ROCK2 is enriched in the brain and heart (Nakagawa et al., 1996).

ROCK is a major downstream effector of the small GTPase RhoA with its C-terminal coiled coil domain specifically interacting with RhoA (Hahmann and Schroeter, 2010; Matsui et al., 1996). The downstream substrates for ROCK include myosin light chain (MLC), MLC phosphatase, ezrin/radixin/moesin, adducin, and LIM kinases, all of which are modulators of actin cytoskeletal organization, stress fiber formation and cell contraction (Nakagawa et al., 1996). The N-terminal serine-threonine kinase domain of ROCK phosphorylates MLC (Leung et al., 1996), which regulates the movement of myosin on actin filaments (Nakagawa et al., 1996). MLC was found to be involved in ULK1-regulated autophagic membrane trafficking (Tang et al., 2010). These findings indicate ULK1 may regulate the autophagosome formation through the Rho GTPase-ROCK-MLC signaling pathway.

V. Summary

In summary, autophagy is a well conserved cellualr degradation pathway engaged in a variety of physiological and pathological conditions. The induction of autophagy in response to nutrition, stress and growth factor regulation is mediated by the ULK1 complex, but the exact mechanism remains unknown. My thesis has aimed to understand the regulation of ULK1 in autophagy.

CHAPTER 2. RESULTS

I. Discovery of MCF2L2 as a novel binding protein of the ULK1 complex

a. Yeast two-hybrid screen identified MCF2L2 as a binding protein of Atg13

To understand how the ULK1 complex regulates the induction of autophagy, we sought to identify other components of the ULK1 complex. According to protein elution analysis in literatures, ULK1 and Atg13 were eluted at a molecular size of 1-3 MDa (Hosokawa et al., 2009a; Mizushima, 2010). Furthermore, several proteins are known as components of the yeast Atg1 complex (Funakoshi et al., 1997; Kabeya et al., 2009), while their corresponding homologues have not been clearly defined in mammalian cells.

In order to search for unknown components in the ULK1 complex, we performed a yeast two-hybrid screen using a human fetal brain cDNA library with Atg13 as bait. We performed two rounds of screens with low and high stringency and obtained four positive hits (Figure 7). The four positive hits included ULK1, suggesting that the yeast two hybrid screen was valid to identify binding partners of Atg13. Two other hits were GATE16 and GABARAP, the LC3-related mammalian homologues of Atg8. A previous study has shown that GATE16 and GABARAP interact with ULK1 (Okazaki et al., 2000), which is consistent with our finding that they are Atg13-binding proteins.

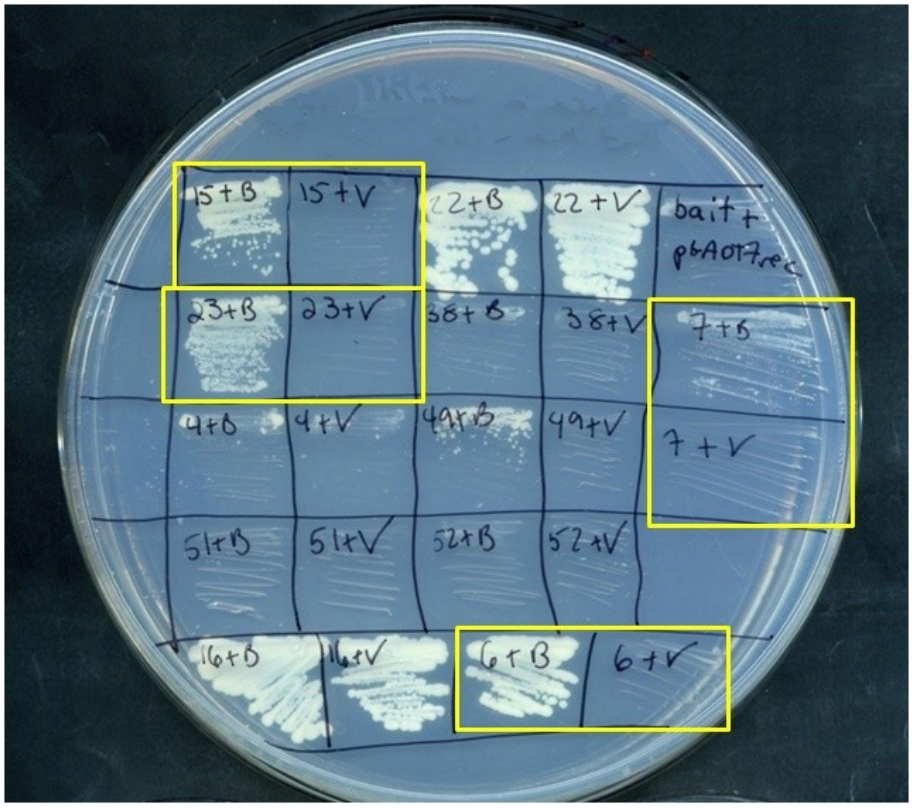


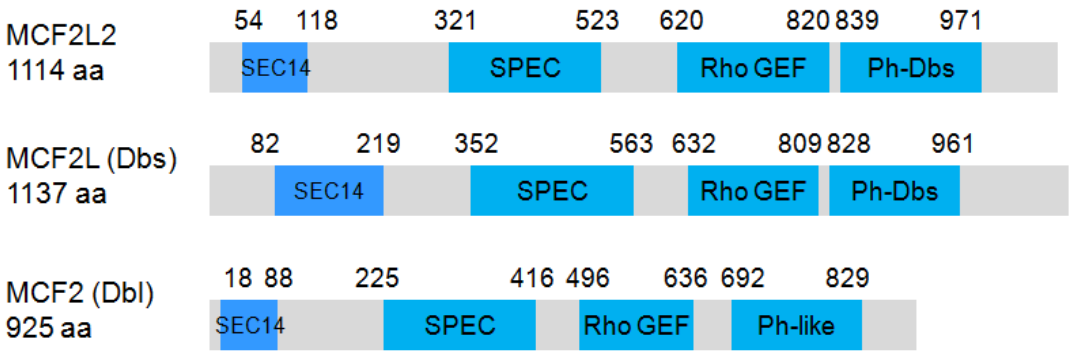
Figure 7. Yeast two-hybrid screening identified 4 potential binding partners of Atg13.

Eleven clones that were selected from a low-stringent screen were tested under a high stringent condition to confirm the interaction with Atg13. The yellow-colored boxes indicate the four positive clones of Atg13-binding proteins that have grown under a high stringent condition (see Materials and Methods section). The clones are: #6, GATE16; #7, GABARAP; #15, ULK1; #23, MCF2L2. Each clone was tested for its growth in the presence of the bait (labeled by "B", which is Atg13) or absence of the bait (labeled by "V", which is vector alone).

The fourth clone contained part (amino acids 540-768) of a gene named *MCF2L2*. Its predicted full length cDNA encodes a protein of 127 kDa with 1114 amino acids. MCF2L2 does not share sequence similarity with any yeast autophagy proteins. MCF2L2 shows 46% of sequence identity and has virtually the identical domains with another protein named MCF2L. MCF2L is also called Dbs (Dbl's big sister), Ost or Rho guanine nucleotide exchange factor-14 (ARHGEF14). Both MCF2L2 and MCF2L are homologous to MCF2 that is also called Dbl (Figure 8a and b). The *MCF2L2* gene exists in higher vertebrates, such as human, chimpanzee, cow, but not found in rodents like mice and rat according to the NCBI protein database (Figure 8c). Interestingly, *MCF2L2* gene was also found in chicken, which are at a lower position in the evolution tree than the rodents (Figure 8c). The *MCF2L* and *MCF2* gene are conserved in a more complete spectrum of vertebrate species from mouse to human, but MCF2 has not been reported in Chimpanzees (Figure 8c). Both MCF2 and MCF2L is known to regulate the small Rho GTPases such as RhoA, Rac1, and CDC42 (Ieguchi et al., 2007). All of these three proteins have four domains: Sec14p-like lipid-binding domain (SEC14); Spectrin repeat motifs (SPEC); RhoGEF domain (or Dbl-homologous (DH) domain; Dbs plecstrin hommology (PH) domain (PH_Dbs) or PH like domain. The SEC14 domain is found in secretory proteins and in lipid regulated proteins such as RhoGAPs and RhoGEFs and it is known to associate with G protein β/γ subunits (Aravind et al., 1999). The SPEC domain is often found in proteins involved in cytoskeletal organization such as spectrin and α-actinin and dystrophin (Broderick and Winder, 2005), and MCF2L2 shares about 50% sequence homology with MCF2K in the domain. The spectrin repeat forms a three-helix bundle (Viel, 1999). The DH domain is invariably present at the N-terminal to the PH domain (Cerione and Zheng, 1996). The PH domain of MCF2 and MCF2L participates in binding to both the Cdc42 and RhoA GTPases (Ieguchi et al., 2007; Whitehead et al., 1999). The specificity of the PH domain is usually determined by the loop regions or insertions at the N-terminal region (Fuentes et al., 2003). The N-terminal regions of the PH domain in MCF2, MCF2L and MCF2L2 are highly conserved (Figure 8b).

While MCF2L is known to regulate small Rho GTPases such as RhoA, Rac1, and CDC42 (Ieguchi et al., 2007), MCF2L2 has not been studied at all for its function. There have been three publications as of August 31, 2012 in the NCBI PubMed database about the *MCF2L2* gene and its variants linked to obesity- and polycystic ovary syndrome (PCOS) (Takeuchi et al., 2008; Zhang et al., 2010; Zheng et al., 2009a). This interesting relationship of MCF2L2 gene to the metabolic diseases has not been explored at all. Since MCF2L2 has been identified as a potential binding protein of Atg13, it is possible that MCF2L2 would affect metabolism through the autophagy process. My thesis work has been focused on confirming the interaction between Atg13 and MCF2L2 and determining the functions of MCF2L2 in the regulation of autophagy.

a



b

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                620                                                 680
MCF2L2          RRRIIRDLLETEEIYIKEIKSIIDGYITPMDFIWLKHLIPDVLQNNKDFLFGNIRELYEF
MCF2L           RRHVMSELLDTERAYVEELLCVLEGYAAEMDNPLMAHLLSTGLHNKKDVLFGNMEEIYHF
MCF2            KNHVLNELIQTERVYVRELYTVLLGYRAEMDNPEMFDLMPPLLRNKKDILFGNMAEIYEF

Prim.cons.      RRHV—ELL-TER-YV-EL—VL-GY-AEMDNP-M-HL-P—L-NKKD-LFGNM-EIYEF

                681                                                 740
MCF2L2          HNRTFLKELEKCAENPELLAHCFLKRKEDLQIYFKYHKNLPRARAIWQECQDCAYFGVCQ
MCF2L           HNRIFLRELENYTDCPELVGRCFLERMEDFQIYEKYCQNKPRSESLWRQCSDCPFFQECQ
MCF2            HNDIFLSSLENCAHAPERVGPCFLERKDDFQMYAKYCQNKPRSETIWRKYSECAFFQECQ

Prim.cons.      HNRIFL-ELENCA—PELVG-CFLERKEDFQIY-KYCQNKPRSE-IWR-CSDCAFFQECQ

                741                                                 800
MCF2L2          RQLDHNLPLFKYLKGPSQRLIKYQMLLKGLLDFESPEDMEIDPGELGGSAKDGPKRTKDS
MCF2L           RKLDHKLSLDSYLLKPVQRITKYQLLLKEML----------------------KYSRNC
MCF2            RKLKHRLRLDSYLLKPVQRITKYQLLLKELL----------------------KYSKDC

Prim.cons.      RKLDH-L-LDSYLLKPVQRITKYQLLLKELLDFESPEDMEIDPGELGGSAKDGPKYSKDC

                801                                                 860
MCF2L2          AFSTELQQALAVIEDLIKSCELAVDLAAVTECPDDIGKLGKLLLHGPFSVWTIHKDRY-K
MCF2L           EGAEDLQEALSSILGILKAVNDSMHLIAITGYDGNLGDLGKLLMQGSFSVWTDHKRGHTK
MCF2            EGSALLKKALDAMLDLLKSVNDSMHQIAINGYIGNLNEIGKMIMQGGFSVWIGHKKGATK
Consensus
Prim.cons.      EGS—LQ-AL—ILDLLKSVNDSMHLIAITGY-GNLG-LGKLLMQG-FSVWT-HK-G-TK

                861                                                 920
MCF2L2          MKDLIRFKPSQRQIYLFERGIVFCKIRMEPGD-QGLSPHYSFKKTMKLMTLSIRQLGRGS
MCF2L           VKELARFKPMQRHLFLHEKAVLFCKKREENGEGYEKAPSYSYKQSLNMAAVGITENVKGD
MCF2            MKDLARFKPMQRHLFLYEKAIVFCKRRVESGEGSDRYPSYSFKHCWKMDEVGITEYVKGD

Prim.cons.      MKDLARFKPMQRHLFL-EKAIVFCK-R-E-GEG——PSYSFK—— KM—VGITE-VKGD

                921
MCF2L2          HRKFEIASRNGLEKYILQAASKEIRDCWFSEISKLLMEQQNNIKDQGNPQFEMS---
MCF2L           AKKFEIWYNAREEVYIVQAPTPEIKAAWVNEIRKVLTSQLQACREASQHRALE----
MCF2            NRKFEIWYGEKEEVYIVQASNVDVKMTWLKEIRNILLKQQELLTVKKRKQQDQLTER

Prim.cons.      -RKFEIWY——EEVYIVQA——EIK—W—EIRK-L—QQ————————Q —— TER
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Rho GEF domain

Ph-Dbs/Ph-like domain

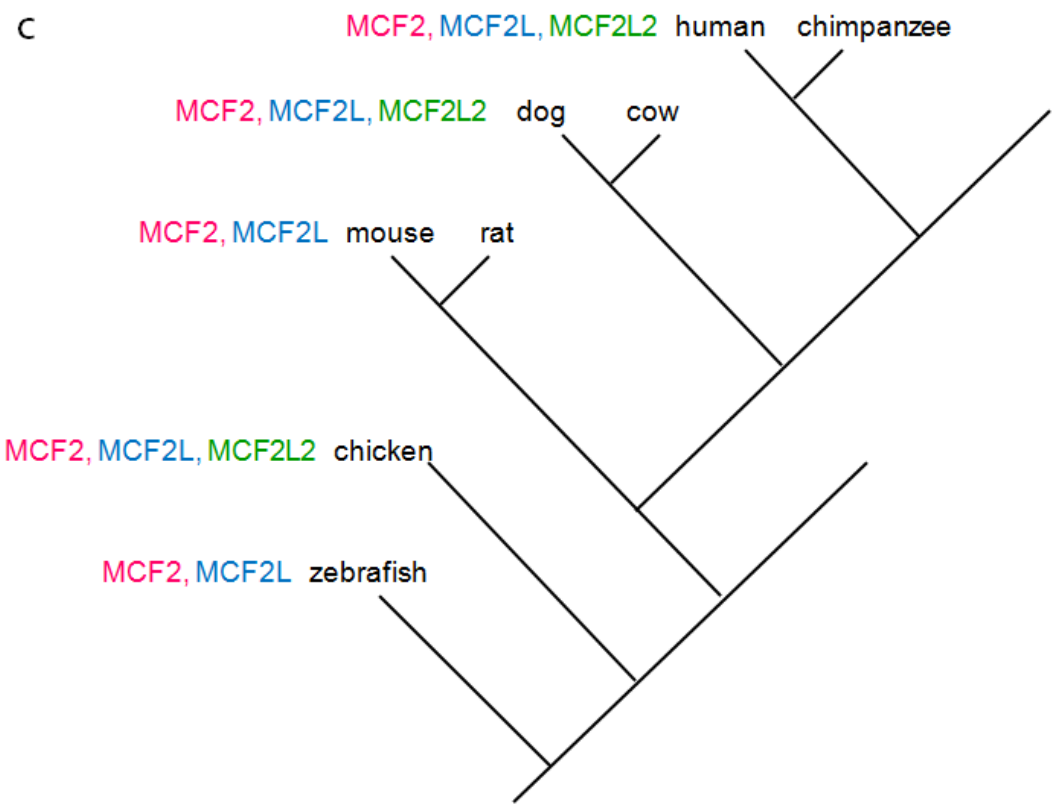


Figure 8. MCF2L2 belongs to the Dbs-related Rho GEF family.

(a) The 3 MCF2L2 homologues contain 4 domains:

SEC14, sec14p-like lipid-binding domain; SPEC, spectrin repeats (SPEC); Rho-GEF, guanine nucleotide exchange factor for Rho/Rac/Cdc42-like GTPases; PH_Dbs, Dbl's big sister (Dbs) pleckstrin homology (PH) domain.

(b) The Rho-GEF domain and the PH_Dbs or PH like domains are highly conserved between MCF2L2, MCF2L and MCF2.

Sequence alignment was made using Multalin program (http://expasy.org/tools/). Primary consensus are listed below the individual sequences.

(c) MCF2L2 gene has not been found in mouse and rat genomes but in chicken, dog, cow, chimpanzee and human genomes (NCBI homologene database).

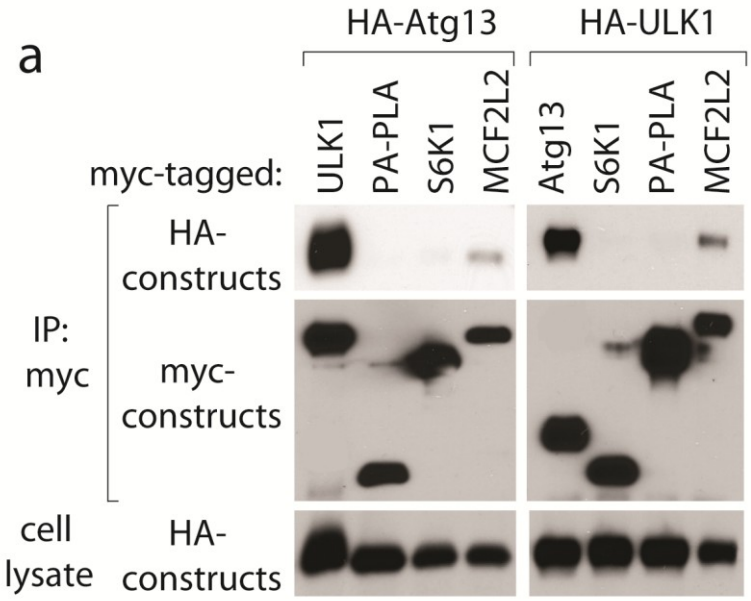
b. **MCF2L2 interacts with the ULK1 complex.**

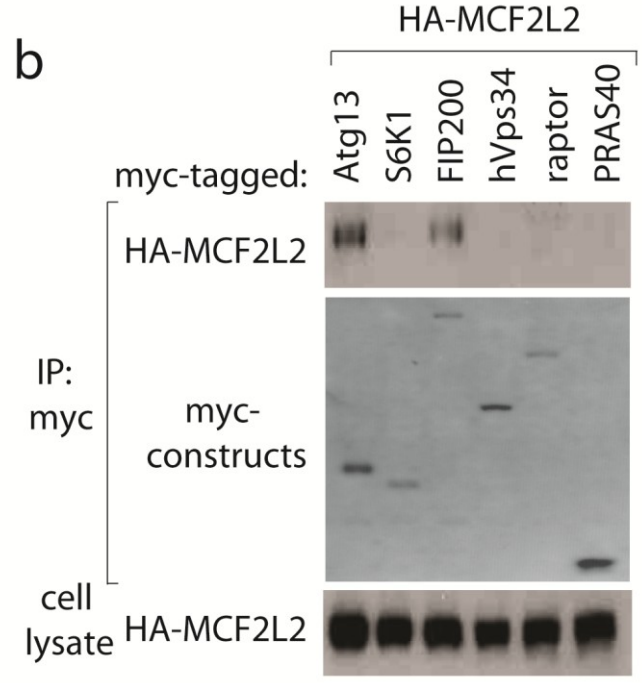
To confirm the result of the yeast two hybrid screen, I first cloned the full length cDNA of *MCF2L2* into prk5-HA mammalian expression vector by PCR amplification. For this, we obtained KIAA0860 and BC064632 clones from Kazusa DNA Research Institute in Japan and Open Biosystems and used them as templates for the PCR cloning. These two clones were missing 5'- and 3'-ends of the full length cDNAs, respectively. Thus, I designed a cloning strategy to combine the two clones using a XhoI site in the middle shared by the two clones. The full-length cDNA of *MCF2L2* gene with 3,349 nucleotides was successfully cloned in the vector and confirmed by sequencing. The cloned full length cDNA was transferred into multiple other expression vectors, including pEGFP-C3, pGEX6P2, and myc-prk5 vectors.

In order to determine if MCF2L2 interacts with Atg13, HEK293T cells were transiently transduced with plasmids encoding myc-MCF2L2 and HA-tagged Atg13. I also included HA-tagged ULK1 to test if MCF2L2 can also interact with ULK1. As negative controls, HA-tagged S6K1 and phosphatidic acid-preferring phospholipase A (PA-PLA) were used. S6K1 and PA-PLA are cytosolic and membrane-bound proteins, respectively. Forty-eight hours post-transfection, cell lysate was obtained and immunoprecipitation was conducted using anti-myc antibody. Immunoprecipitated complexes were run on a SDS-PAGE and analyzed by Western blotting. Both HA-tagged ULK1 and Atg13 were detected in the immunoprecipitate isolated with myc-tagged MCF2L2 (Figure 9a). I could not detect any control protein immunoprecipitated with myc-MCF2L2. I also tested FIP200, another component of the ULK1 complex, for its interaction with MCF2L2. For this experiment, I included multiple negative control proteins, such as S6K1, hVps34, raptor and PRAS40, as well as Atg13. HA-MCF2L2 was co-immunoprecipitated with myc-tagged FIP200 and Atg13 but not with the negative control proteins (Figure 9b). Combined, these results suggest that MCF2L2 interacts with the ULK1 complex.

To confirm if the interaction can occur between endogenous proteins, we generated polyclonal antibodies against human MCF2L2. The C-terminal fragment of MCF2L2 containing amino acids 250-1129 was expressed as a N-terminal glutathione S-transferase (GST)-fusion form in bacteria and purified through GSH affinity column. The purified fragment was used to generate polyclonal antibodies in rabbit. The generated antibody was confirmed to recognize endogenous MCF2L2 specifically (Figure 9c).

To confirm the interaction between endogenous proteins using the antibody, Atg13 immunoprecipitate was obtained using anti-Atg13 antibody from HEK293T cells and the presence of MCF2L2 in the immunoprecipitate was analyzed by Western blotting. As a control, pre-immune serum was used instead of Atg13 antibody. MCF2L2 was detected with the immunoprecipitate obtained using anti-Atg13 antibody but not pre-immune serum (Figure 9d). Inversely, ULK1 and Atg13 were detected in immune complex obtained using anti-MCF2L2 antibody but not pre-immune serum (Figure 9e). Collectively, these results suggest that MCF2L interacts with the ULK1 complex at recombinant and endogenous levels.


a
HA-Atg13
HA-ULK1
myc-tagged:
ULK1
PA-PLA
S6K1
MCF2L2
Atg13
S6K1
PA-PLA
MCF2L2
IP: myc
HA-constructs
myc-constructs
cell lysate
HA-constructs


b
HA-MCF2L2
myc-tagged:
Atg13
S6K1
FIP200
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raptor
PRAS40
IP: myc
HA-MCF2L2
myc-constructs
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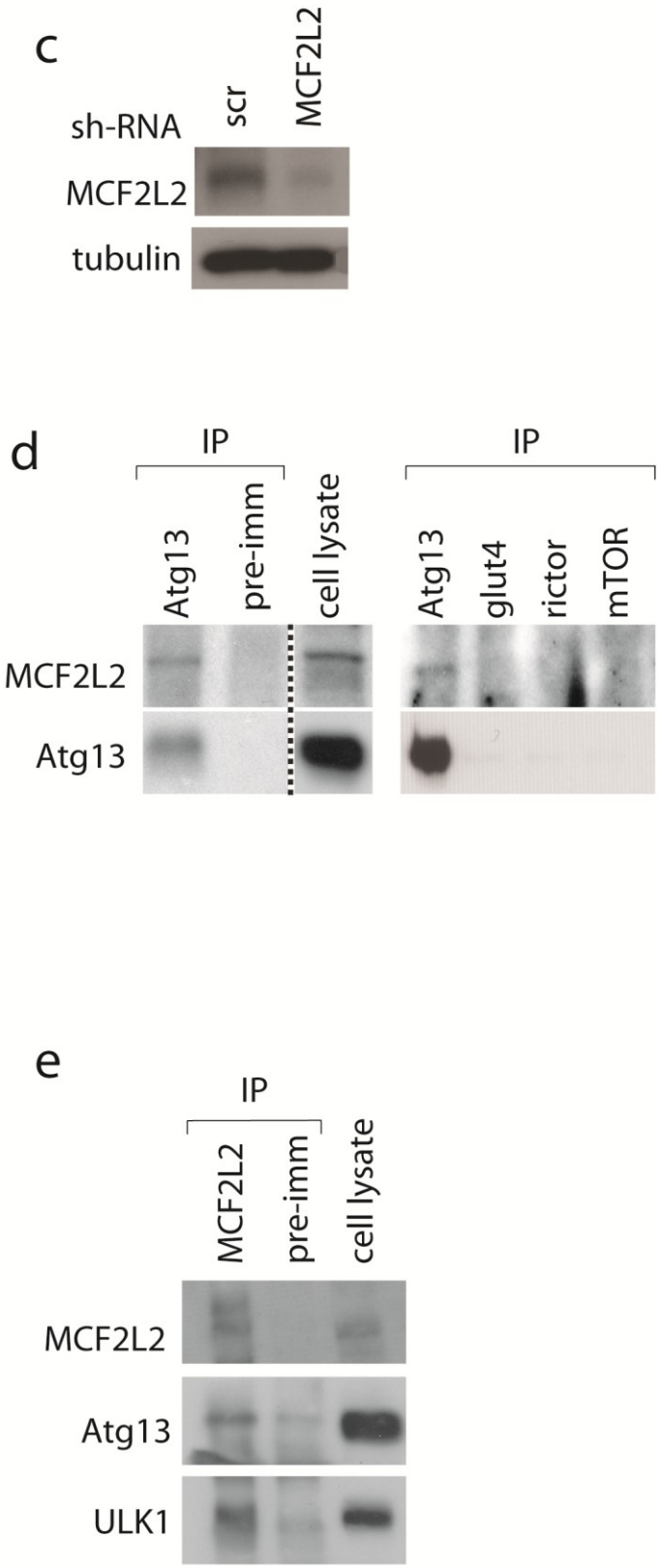
HA-MCF2L2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IP
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glut4
rictor
mTOR
MCF2L2
Atg13
e
IP
MCF2L2
pre-imm
cell lysate
MCF2L2
Atg13
ULK1

Figure 9. MCF2L2 interacts with ULK1, Atg13 and FIP200.

(a) ULK1 and Atg13 are coimmunoprecipitated with MCF2L2.

HA-tagged ULK1 or Atg13 was expressed with myc-tagged MCF2L2 or control proteins (S6K1 and PA-PLA as negative controls, and Atg13 or ULK1 as positive control) in HEK293T cells. Anti-myc immune complexes were assessed for the presence of HA-ULK1 and HA-Atg13 by Western blotting.

(b) MCF2L2 binds ULK1, Atg13 and FIP200.

HA-tagged MCF2L2 was expressed with myc-tagged ULK1, Atg13, FIP200 or control proteins (hVps34, raptor, PRAS40) in HEK293T cells. Anti-myc immune complexes were assessed for the presence of HA-MCF2L2 by Western blotting.

(c) MCF2L2 antibody specifically binds to endogenous MCF2L2 .

HEK293 cells were transduced with sh-RNA for MCF2L2 or scramble as control. Cell lysate was assayed by Western blotting for the presence of MCF2L2 (~127 kD) with MCF2L2 antibody.

(d) Endogenous MCF2L2 is coimmunoprecipitated with Atg13.

Atg13 immunoprecipitate using anti-Atg13 serum was obtained from HEK293T cells and the amounts of MCF2L2 in the immune complexes were assayed by Western blotting. Atg13 pre-immune serum immunoprecipitate was assayed as negative control. Atg13 immunoprecipitate using anti-Atg13 antibody was obtained from HEK293T cells too and the amounts of MCF2L2 in the immune complexes were assayed by Western blotting. Glut4, rictor, and mTOR antibodies were used as negative controls.

(e) Endogenous ULK1 and Atg13 interact with MCF2L2.

MCF2L2 immunoprecipitate was obtained from HEK293T cells and the amounts of ULK1 and Atg13 in the immune complexes were assayed by Western blotting. MCF2L2 pre-immune serum immunoprecipitate was assayed as negative control.

c. MCF2L2 directly binds to Atg13 independently of ULK1.

The background section introduced that ULK1 is a multi-functional protein with its role not limited to autophagy. Its N-terminal region contains a kinase domain while the C-terminal region interacts with different binding partners. When interacting with Atg13, ULK1 was found to function in autophagy, while when interacting with other proteins, ULK1 may switch to function in neuronal regulation (Lai and Garriga, 2004; Ogura et al., 1997). Therefore, it is important to determine if MCF2L2 binds to ULK1 through Atg13 so it functions in autophagy pathway.

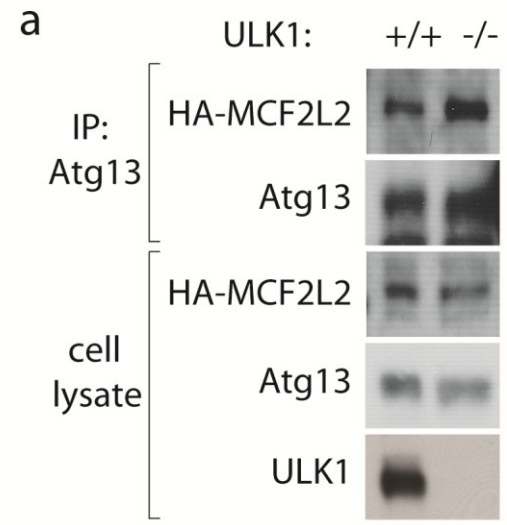
To determine if the interaction between Atg13 and MCF2L2 depends on ULK1, I analyzed the interaction in ULK1-deficient MEFs. Since MEFs do not express MCF2L2, I transiently expressed HA-MCF2L2 in MEFs. I isolated immune complex using anti-Atg13 antibody and analyzed the amount of MCF2L2 in the immune complex. MCF2L2 was still detected in the anti-Atg13 immune complex isolated from ULK1-deficient MEFs (Figure 10a), suggesting that Atg13 can bind to MCF2L2 independently of ULK1. Next, I used Atg13-silenced HEK293T cells to determine if Atg13 is important for the interaction between MCF2L2 and ULK1. Knockdown of Atg13 in HEK293T cells almost completely abolished the interaction between ULK1 and MCF2L2 (Figure 10b). This result suggests that Atg13, but not ULK1, is important for the binding of MCF2L2 to the ULK1 complex. The dependence of the interaction on Atg13 implies that MCF2L2 might participate in autophagy rather than non-autophagic functions mediated by ULK1.

d. N-terminal region of MCF2L2 binds to a C-terminal region of ULK1 through Atg13.

To better understand how MCF2L2 interacts with the ULK1 complex, I conducted a delimitation experiment to identify the region of MCF2L2, Atg13 and ULK1 involved in the interaction. It is known that ULK1 interacts with Atg13 via its C-terminal

region (Hosokawa et al., 2009b). Since Atg13, but not ULK1, binds to MCF2L2, I predicted that MCF2L2 might bind to the C-terminal region of ULK1 where Atg13 binds. As predicted, I found that MCF2L2 binds to the C-terminal region of ULK1 containing amino acid residues 651-1051 (Figure 10c). The binding of MCF2L2 to the C-terminal region of ULK1 further confirms that MCF2L2 binds to ULK1 via Atg13. I found that all the fragments of Atg13 can interact with MCF2L2 (Figure 10d). A possible explanation might be that Atg13 might require its whole sequence for binding to MCF2L2 or multiple regions of Atg13 might be involved in the interaction with MCF2L2. I also performed a delimitation experiment to determine which region(s) of MCF2L2 are involved in the interaction of MCF2L2 with Atg13. I found that Atg13 binds to a N-terminal region of MCF2L2 within amino acids 1-500 (Figure 10e).

To confirmed if MCF2L2 and Atg13 directly interact with each other, I attempted to purify MCF2L2 from bacteria. Since the full length MCF2L2 was not able to be purified in a soluble form, we purified its fragments. Knowing that the N-terminal region of MCF2L2 interacts with Atg13 (Figure 10e), I expressed and purified N-terminal fragments of MCF2L2 and successfully obtained their soluble forms. Through GSH pull down experiment using the purified fragments, I confirmed that MCF2L2 can interact with Atg13 *in vitro* (Figure 10f). Collectively, these results suggest that a N-terminal region of MCF2L2 directly interacts with Atg13.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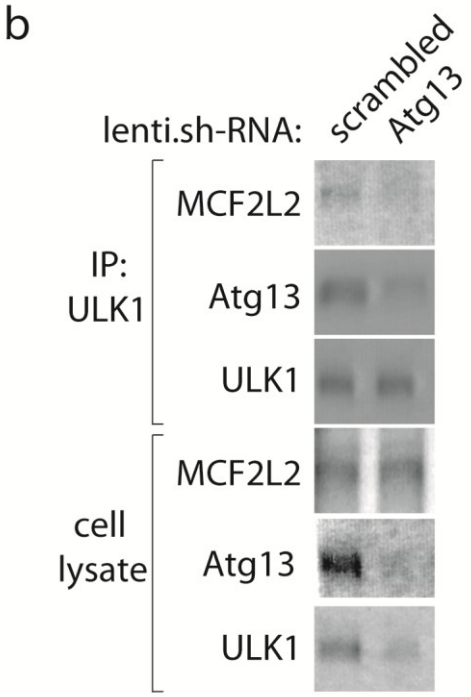
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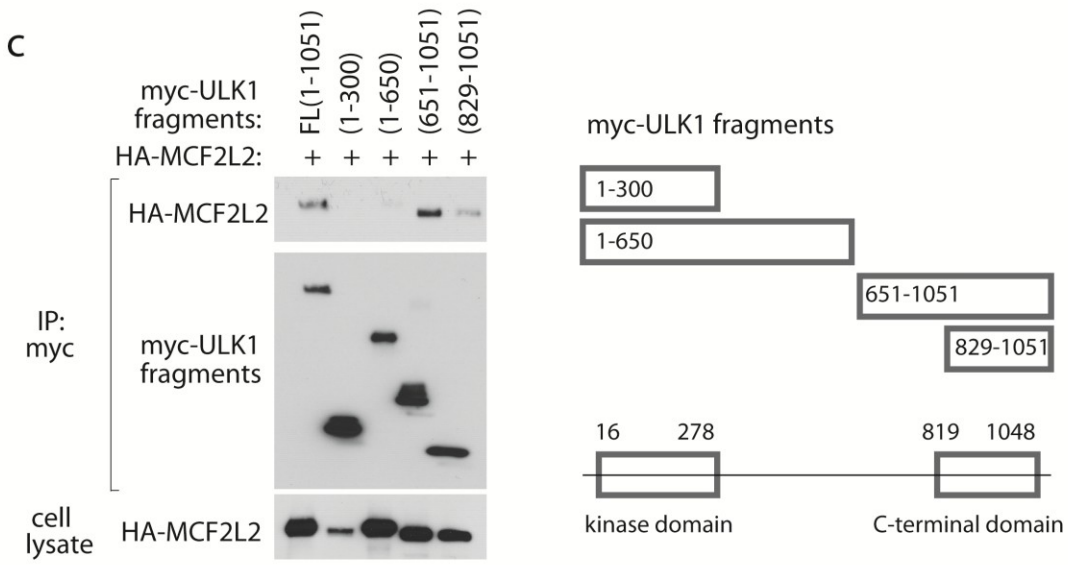
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(1-650)
(651-1051)
(829-1051)
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HA-MCF2L2
IP: myc
myc-ULK1 fragments
cell lysate
HA-MCF2L2
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16
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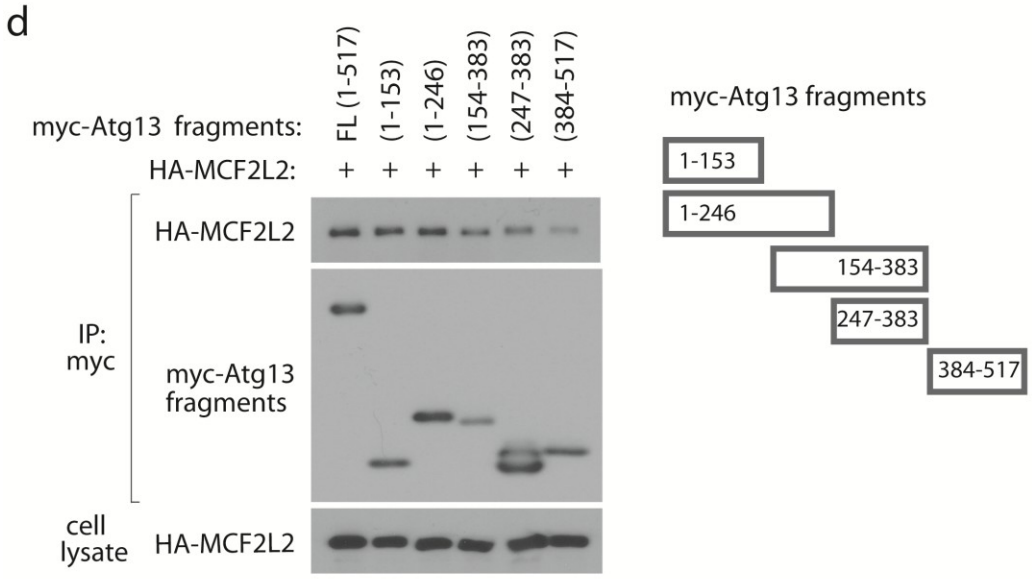
kinase domain
C-terminal domain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(247-383)
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HA-MCF2L2
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myc-Atg13 fragments
1-153
1-246
154-383
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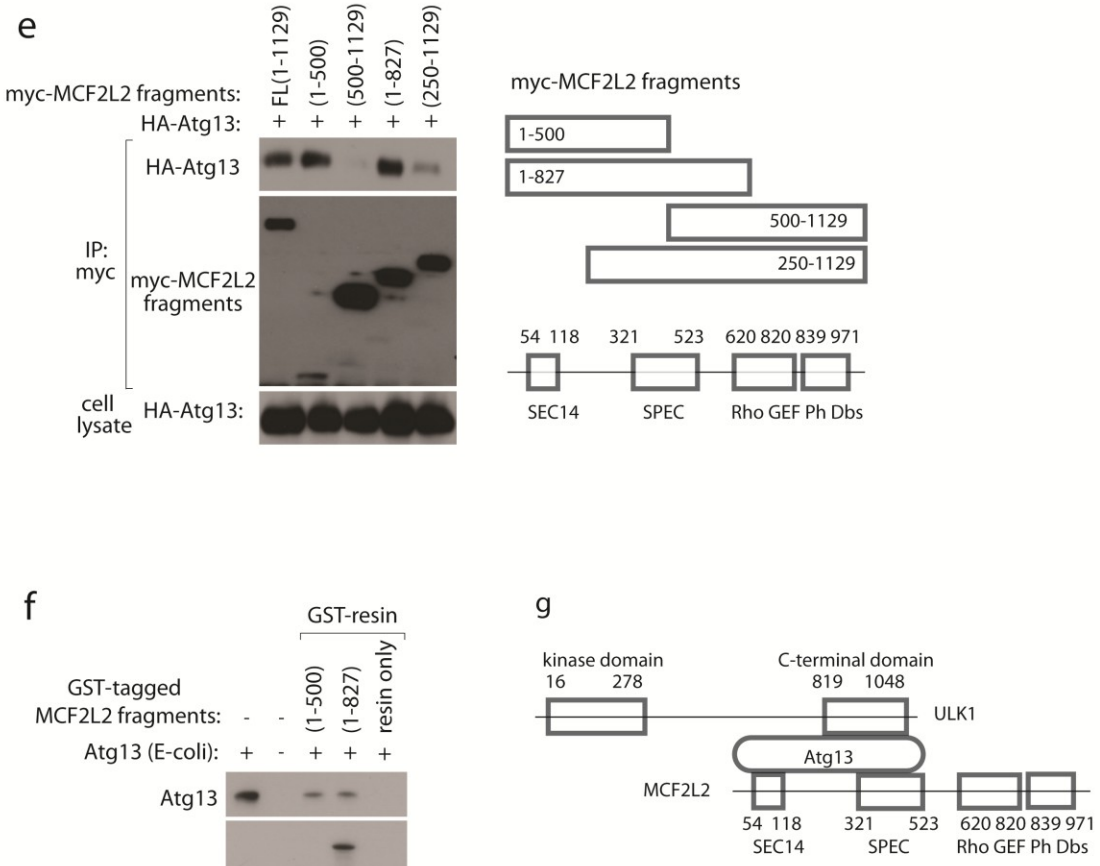
384-517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HA-Atg13
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myc-MCF2L2 fragments
cell lysate
HA-Atg13:
myc-MCF2L2 fragments
1-500
1-827
500-1129
250-1129
54 118 321 523 620 820 839 971
SEC14
SPEC
Rho GEF Ph Dbs
f
GST-resin
GST-tagged MCF2L2 fragments: - - (1-500) (1-827) resin only
Atg13 (E-coli): + - + + +
Atg13
GST-constructs
g
kinase domain
16 278
C-terminal domain
819 1048
ULK1
Atg13
MCF2L2
54 118
SEC14
321 523
SPEC
620 820 839 971
Rho GEF Ph Dbs

Figure 10. Atg13 directly binds to MCF2L2.

(a) MCF2L2 can interact with Atg13 in the absence of ULK1.

HA-tagged MCF2L2 was expressed in ULK1 deficient (-/-) or wild type (+/+) MEFs. Anti-Atg13 immune complexes were obtained and analyzed for the presence of HA-MCF2L2 by Western blotting.

(b) Atg13 is necessary for the interaction between ULK1 and MCF2L2.

ULK1 immunoprecipitate was obtained from HEK293T cells transduced with either scrambled shRNA or Atg13 shRNA. The amount of MCF2L2 in the ULK1 immune complex was analyzed by Western blotting.

(c) MCF2L2 interacts with ULK1 C-terminal region.

Myc-tagged fragments of ULK1 were coexpressed with HA-tagged MCF2L2 in HEK293T cells. The amount of HA-MCF2L2 in myc immunoprecipitate was analyzed on Western blots.

(d) MCF2L2 requires the entire region of Atg13 for binding.

Myc-tagged fragments of Atg13 were coexpressed with HA-tagged MCF2L2 in HEK293T cells. The amount of HA-MCF2L2 in anti-myc immune complex was analyzed by Western blotting.

(e) Atg13 interacts with MCF2L2 N-terminal region.

Myc-tagged fragments of MCF2L2 were coexpressed with HA-tagged Atg13 in HEK293T cells. The amount of HA-Atg13 in myc immunoprecipitate was analyzed on Western blots.

(f) Atg13 directly interacts with MCF2L2 N-terminal regions.

GST alone or GST-tagged MCF2L2 N-terminal fragments were incubated with *bacterial*–purified Atg13, and the amounts of Atg13 recovered with GST-tagged proteins were analyzed by Western blotting.

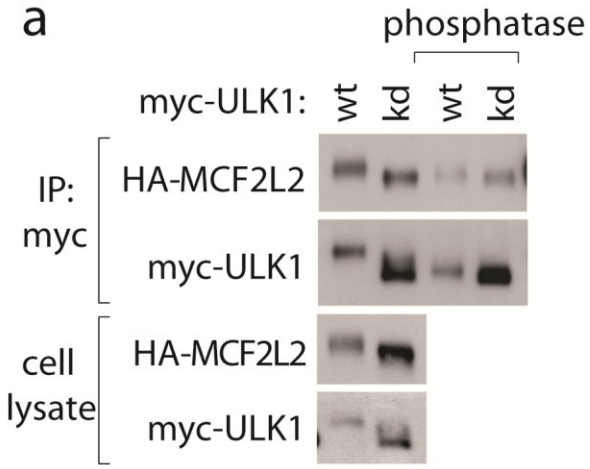
(g) Schematic model of the interaction between MCF2L2, ULK1 and Atg13.

It is proposed that the N-terminal region of MCF2L2 interacts with Atg13 and binds to the C-terminal region of ULK1.

e. MCF2L2 is a potential substrate of ULK1.

Previous studies have shown that ULK1 phosphorylates Atg13, FIP200 and itself (Hosokawa et al., 2009a; Jung et al., 2009). Thus, I tested if MCF2L2 would also be phosphorylated by ULK1. Supporting this possibility, I found that wild type but not kinase dead mutant form of ULK1 coexpressed with HA-MCF2L2 induced a upward shift of HA-MCF2L2 protein band in SDS-PAGE. The mobility shift was due to phosphorylation, since it was almost completely suppressed by treatment with λ phosphotase (Figure 11a). The induced upward shift by wild type ULK1 was shown with the fragments containing amino acids 500-1129 and amino acids 250-1129 but not amino acids 1-827 (Figure 11b). This implies that the ULK1 phosphorylation sites in MCF2L2 might lie in the C-terminal region containing amino acids 828-1129.

To determine if ULK1 can directly phosphorylate MCF2L2, I conducted *in vitro* kinase assay using purified MCF2L2 fragments and immunoprecipitated ULK1 kinase as the enzyme source. The *In vitro* kinase assay revealed that the C-terminal fragments of MCF2L2 can be directly phosphorylated by ULK1 (Figure 11c). As a control, the kinase-dead mutant form of ULK1 did not phosphorylate MCF2L2 (Figure 11c). Combined, these results suggest that ULK1 has the capacity to phosphorylate MCF2L2.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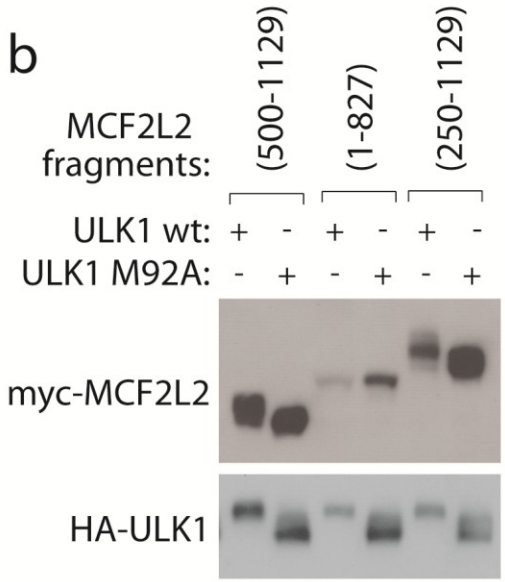
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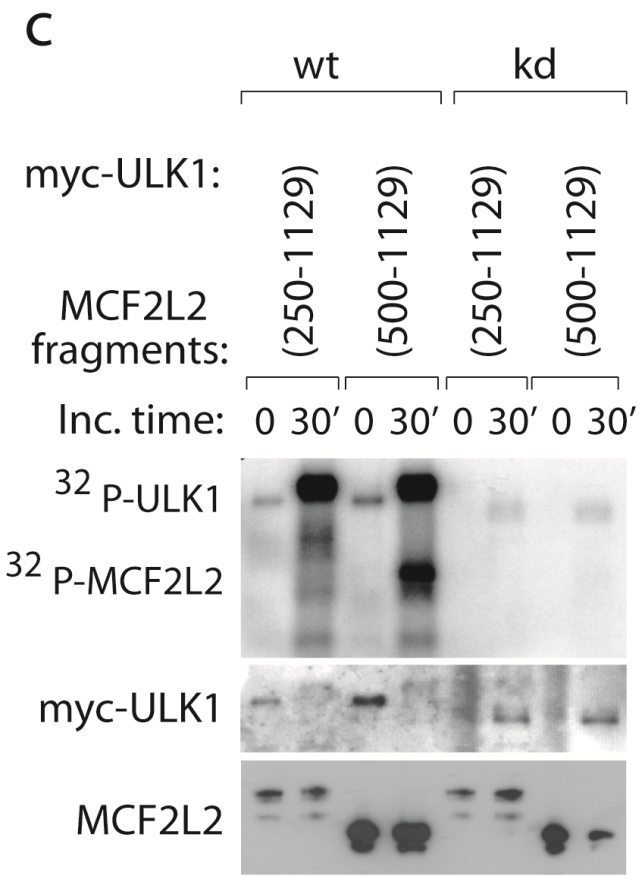


Figure 11. ULK1 phosphorylates MCF2L2.

(a) ULK1 induces the phosphorylation of MCF2L2.

HA-tagged MCF2L2 was coexpressed with myc-tagged wild type (wt) or kinase dead (kd) mutant form of ULK1 in HEK293T cells. Myc immunoprecipitate was obtained and treated with or without λ phosphatase for 30 min. The migration patterns of MCF2L2 and ULK1 isolated from myc immunoprecipitation were analyzed on SDS-PAGE.

(b) ULK1 induces the phosphorylation of MCF2L2 in a region near C-terminus.

Myc-tagged MCF2L2 fragments were coexpressed with HA-tagged wt or kd mutant form of ULK1 in HEK293T cells. The migration patterns of MCF2L2 fragments and ULK1 were analyzed on SDS-PAGE.

(c) ULK1 directly phosphorylates MCF2L2 *in vitro*.

Myc-tagged wt or kd mutant form of ULK1 was isolated by myc immunoprecipitation from HEK293T cells and incubated with MCF2L2 C-terminal fragments purified from bacteria in the presence of ^{32}P-ATP *in vitro*. The incorporation of ^{32}P into ULK1 and MCF2L2 fragments was analyzed by autoradiography.

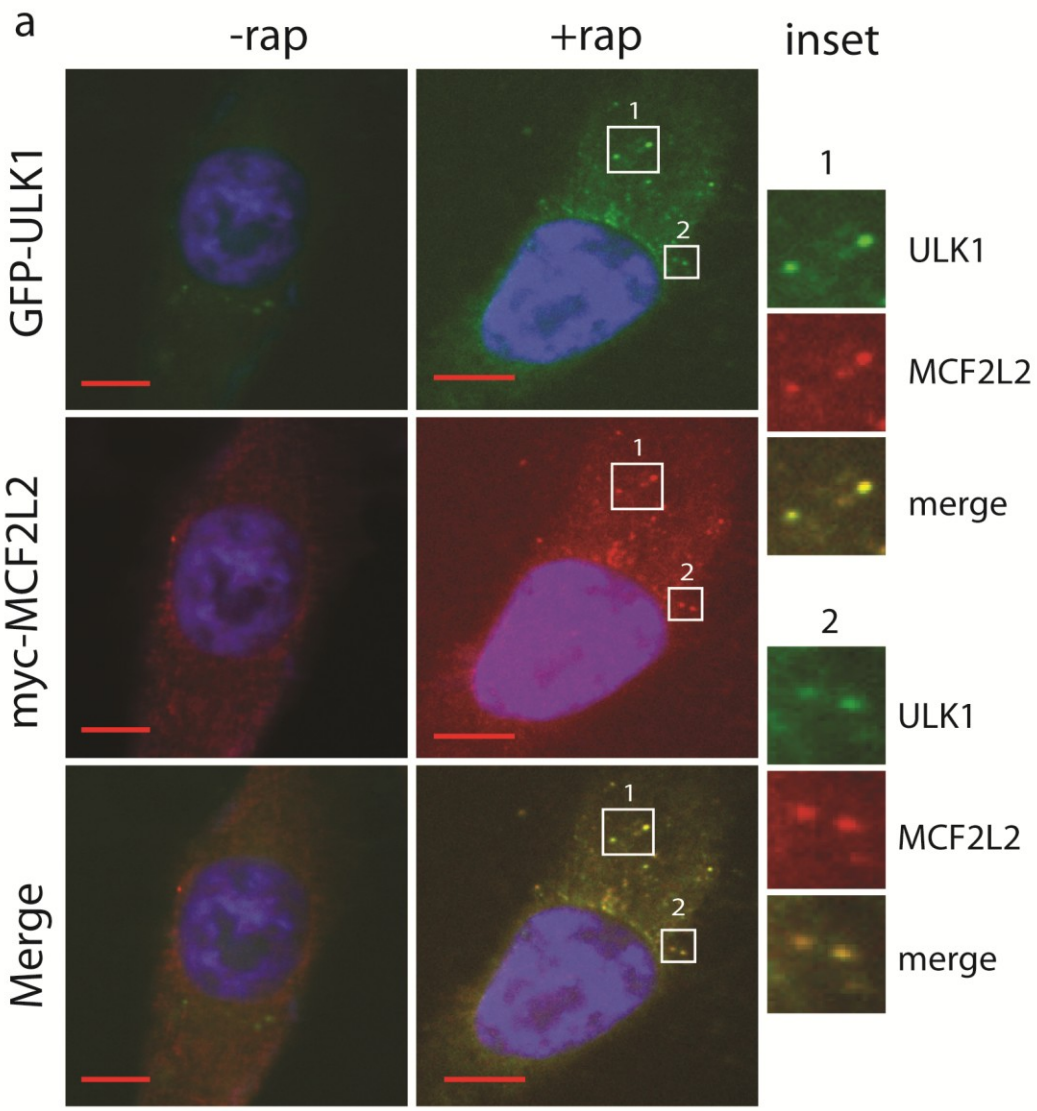
II. MCF2L2 plays an important role in autophagy induction

a. MCF2L2 knockdown suppressed autophagy.

Together with the finding that MCF2l2 directly interacts with Atg13, the finding that MCF2L2 is a potential substrate of ULK1 added to the evidence that MCF2L2 may be an essential component in the ULK1-mediated autophagy induction pathway. Previous studies have shown that ULK1 forms puncta structures in response to starvation (Hara et al., 2008; Itakura and Mizushima, 2010). Since MCF2L2 is a component of the ULK1 complex, I tested if MCF2L2 can also form puncta structure. In HeLa cells, GFP-tagged ULK1 formed puncta structures upon rapamycin treatment, and the GFP-ULK1 puncta co-localized with myc-MCF2L2 puncta (Figure 12a). This result suggests that MCF2L2, as a component of the ULK1 complex, might participate in the formation of the ULK1 puncta or the formation of isolation membrane (Hara et al., 2008; Itakura and Mizushima, 2010).

In order to better understand the function of MCF2L2 in autophagy, I have generated HeLa and HEK293 cells where MCF2L2 was stably silenced by lentiviral shRNA. Using the stable cells, I first analyzed if MCF2L2 knockdown affects the autophagosome formation by staining endogenous LC3 in the shRNA-transduced HeLa cells. Rapamycin for 4 hrs induced LC3-positive puncta or autophagosome in GFP shRNA-transduced HeLa cells (Figure 12b). The formation of autophagosome was suppressed in the cells stably transduced by MCF2L2 shRNA (Figure 12b and c). This result suggests that MCF2L2 is important for autophagosome formation. To further confirm the role of MCF2L2 in autophagy, autophagy flux was assayed by measuring the levels of LC3-I and LC3-II in the presence or absence of lysosomal inhibitors. In control cells, treatment of lysosomal inhibitors such as E64 and pepstatin dramatically enhanced the accumulation of LC3-II when cells were simultaneously treated with rapamycin (Figure 11d). By contrast, MCF2L2-silenced cells did not show the prominent increase in LC3-II level (Figure 12d). I also found that LC3-I, the cytosolic form of LC3,

accumulated in cells with MCF2L2 silenced, suggesting that the autophagy flux was blocked in these cells (Figure 12d). The degradation of p62 is mainly dependent upon the autophagy process (Mizushima and Yoshimori, 2007). I found that rapamycin treatment decreased the amount of p62 in control cells, whereas the decrease in p62 was suppressed in MCF2L2-silenced cells (Figure 11c). Combined, these results suggest that MCF2L2 is involved in autophagy process.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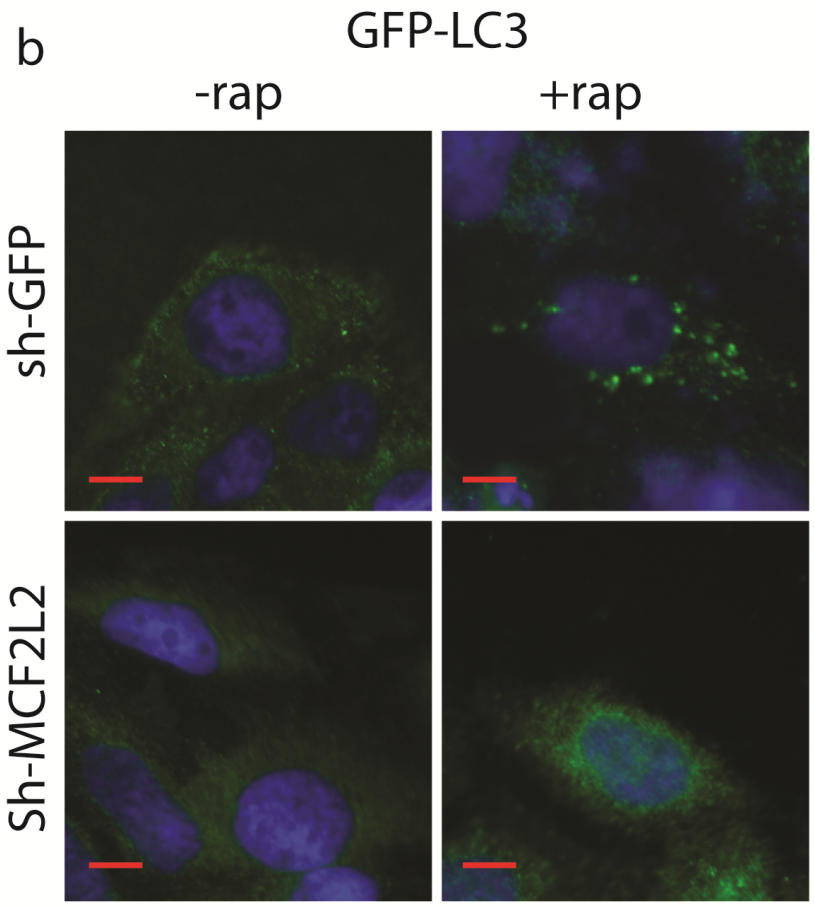
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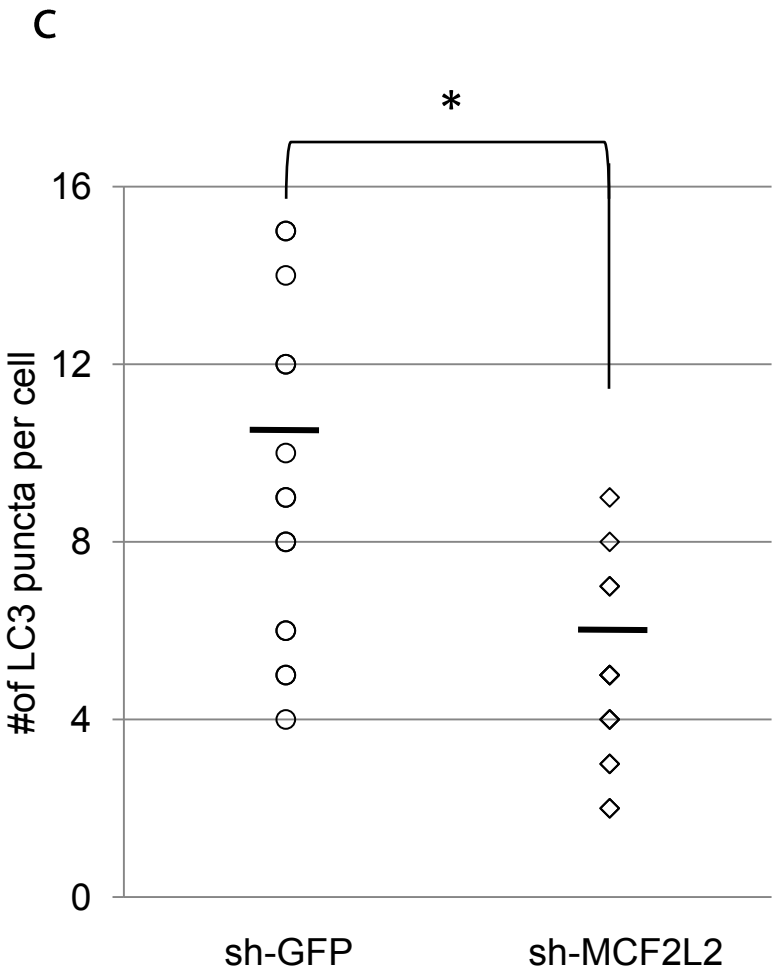
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8
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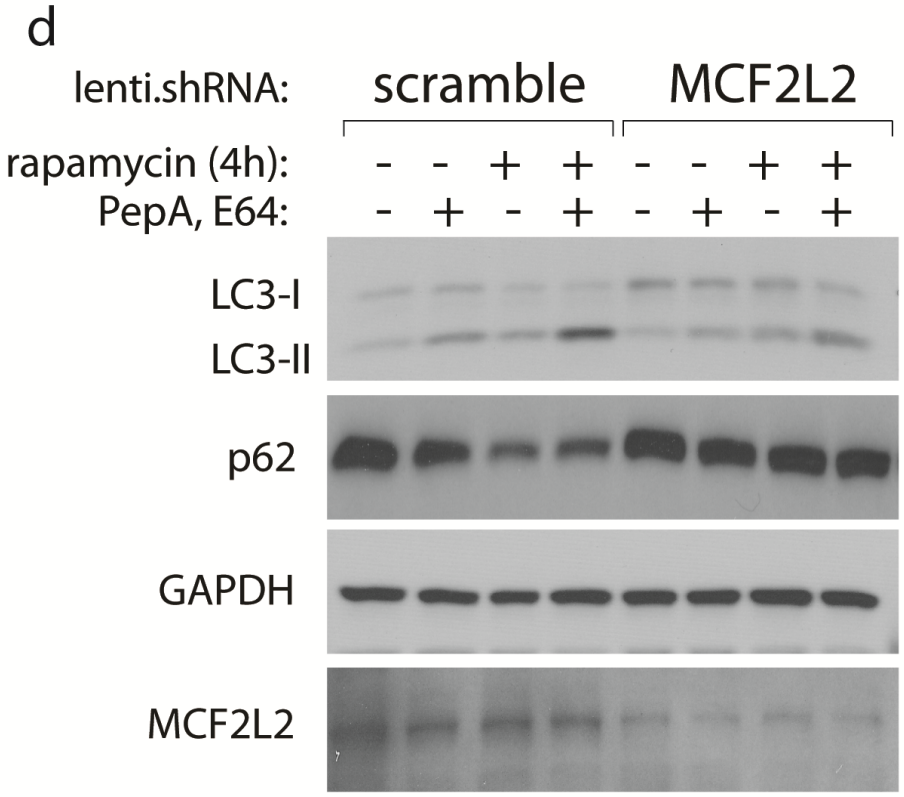
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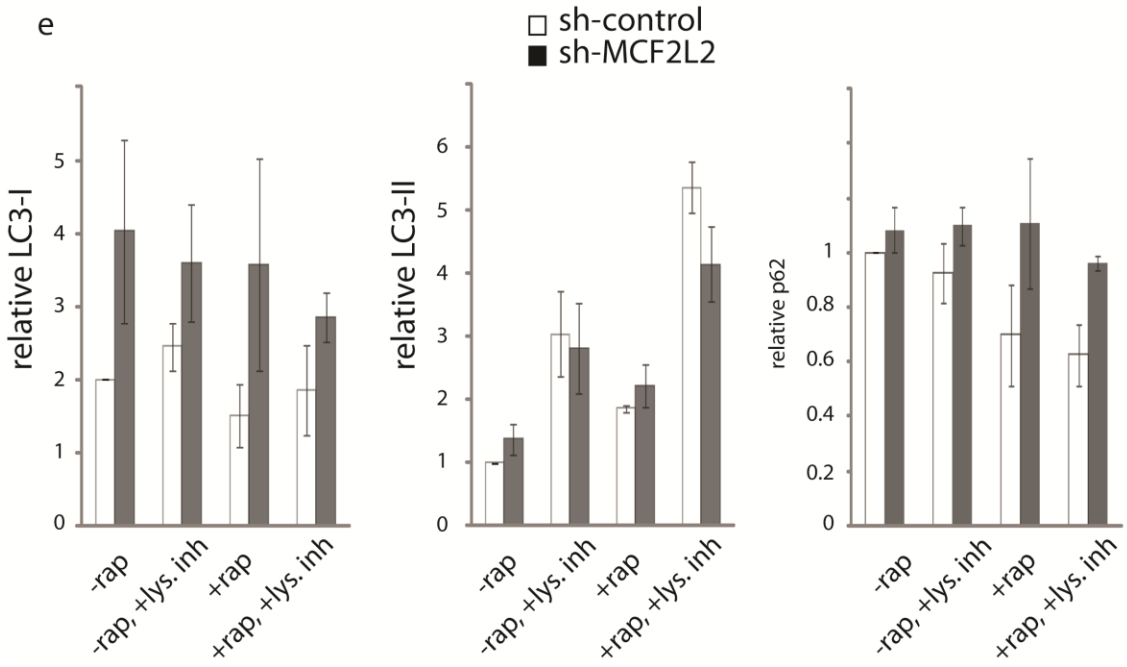


Figure 12. MCF2L2 is important for autophagy.

(a) MCF2L2 forms punctual structures and co-localizes with ULK1 puncta in response to rapamycin.

Twenty four hours post-transfection with GFP-tagged ULK1 and myc-MCF2L2, HeLa cells were treated with 100 nM rapamycin or dimethyl sulfoxide (DMSO, vehicle) for 2 hrs. Cells were stained using myc antibody conjugated with Alexa 647 (red) and visualized by microscope (Olympus Fluoview 1000 IX2 inverted microscope). Colocalization of ULK1 puncta and MCF2L2 puncta is shown in yellow color. (Inset) Higher magnification demonstrates co-localization of ULK1 puncta and MCF2L2 puncta in rapamycin-treated cells. Nuclei were stained with DAPI (blue). Scale bar,10 μm.

(b) Knockdown of MCF2L2 inhibits the formation of LC3-positive autophagosomes. HeLa cells stably transduced by lentiviral shRNA were treated with rapamycin (100 nM) or DMSO for 4 hrs. Cells were stained with anti-LC3 antibody (green) and visualized by confocal microscope. Nuclei were stained with DAPI (blue). Scale bar,10 μm.

(c) Quantitative analysis of GFP-LC3 puncta per cell (*$p < 0.05$ from Student's t test, n = 15). Mean value is shown as a horizontal bar

(d) Knockdown of MCF2L2 inhibits rapamycin-induced autophagic flux of LC3 and suppresses the degradation of p62.

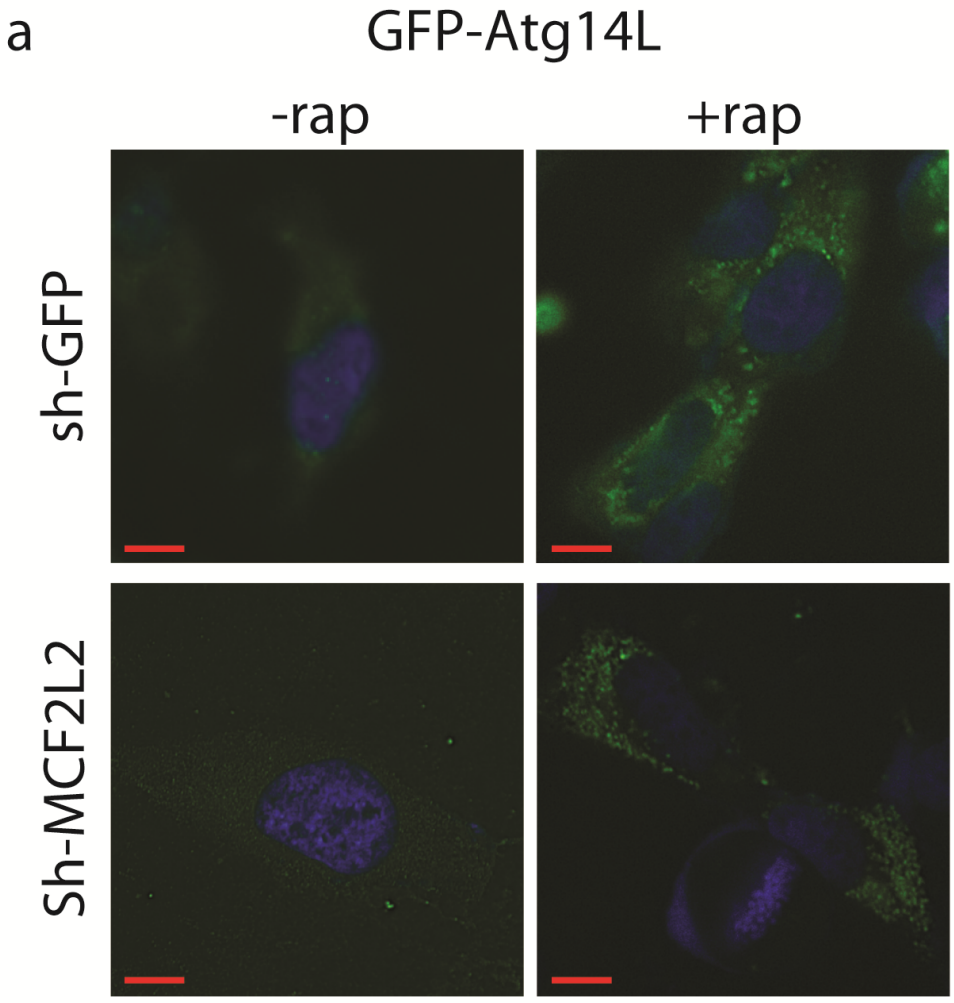
HEK293T cells stably transduced by lentiviral shRNA for MCF2L2 or scramble shRNA were treated with rapamycin (100 nM) or DMSO for 4 h in the presence or absence of lysosomal inhibitors (LI), pepstatin A (10 μg/ml) and E-64 (10μg/ml), and the indicated proteins in cell lysates were analyzed by Western blotting. The level of GAPDH is shown as loading control.

(e) Quantification of the western blot band density for autophagy flux markers, P62, LC3-I and LC3-II.

b. MCF2L2 acts downstream of ULK1 to regulate the production of PI3P essential for the formation of the isolation membrane

Knowing that MCF2L2 is involved in autophagy, I investigated further which step MCF2L2 regulates in the autophagy pathway. As explained early in the Background section, the ULK1 complex regulates the early step of the isolation membrane formation (Hosokawa et al., 2009a; Itakura and Mizushima, 2010). There are two events that were reported to be closely related to the ULK1 puncta formation. First, it has been shown that the ULK1 complex forms punctuate structure together with the Atg14L-containing PI3KC3 complex upon autophagy induction (Itakura and Mizushima, 2010). I found that the formation of Atg14L puncta was not affected by MCF2L2 knockdown (Figure 13a). Thus, MCF2L2 is unlikely involved in the regulation of Atg14L puncta formation that is dependent upon the ULK1 complex.

Second, the ULK1 puncta co-localize with the isolation membrane based on they colocalization with WIPIs, the downstream effector of PI3KC3 and markers for the isolation membrane (Itakura and Mizushima, 2010). The generation of PI3P and the puncta formation of WIPIs on the isolation membrane have been shown to depend on the ULK1 complex (Itakura and Mizushima, 2010). I tested if MCF2L2 is important for the formation of WIPI-1 or ULK1 puncta using MCF2L2-silenced HeLa cells. I found that the majority of WIPI-1 puncta colocalized with ULK1 puncta in GFP shRNA-transduced control cells. Silencing of MCF2L2 did not affect the puncta formation of GFP-ULK1, but disrupted the co-localization of ULK1 puncta with WIPI-1 puncta (Figure 13b and 13c). This result suggests that MCF2L2 might be important for the ULK1 complex to recruit WIPI-1 in the formation of the isolation membrane. Since MCF2L2 did not affect Atg14L puncta formation, the result indicates that MCF2L2 might be important for events downstream of the Atg14L-containing PI3KC3 complex, maybe the activation of PI3KC3 complex or the recruitment and transport of WIPIs to the isolation membrane.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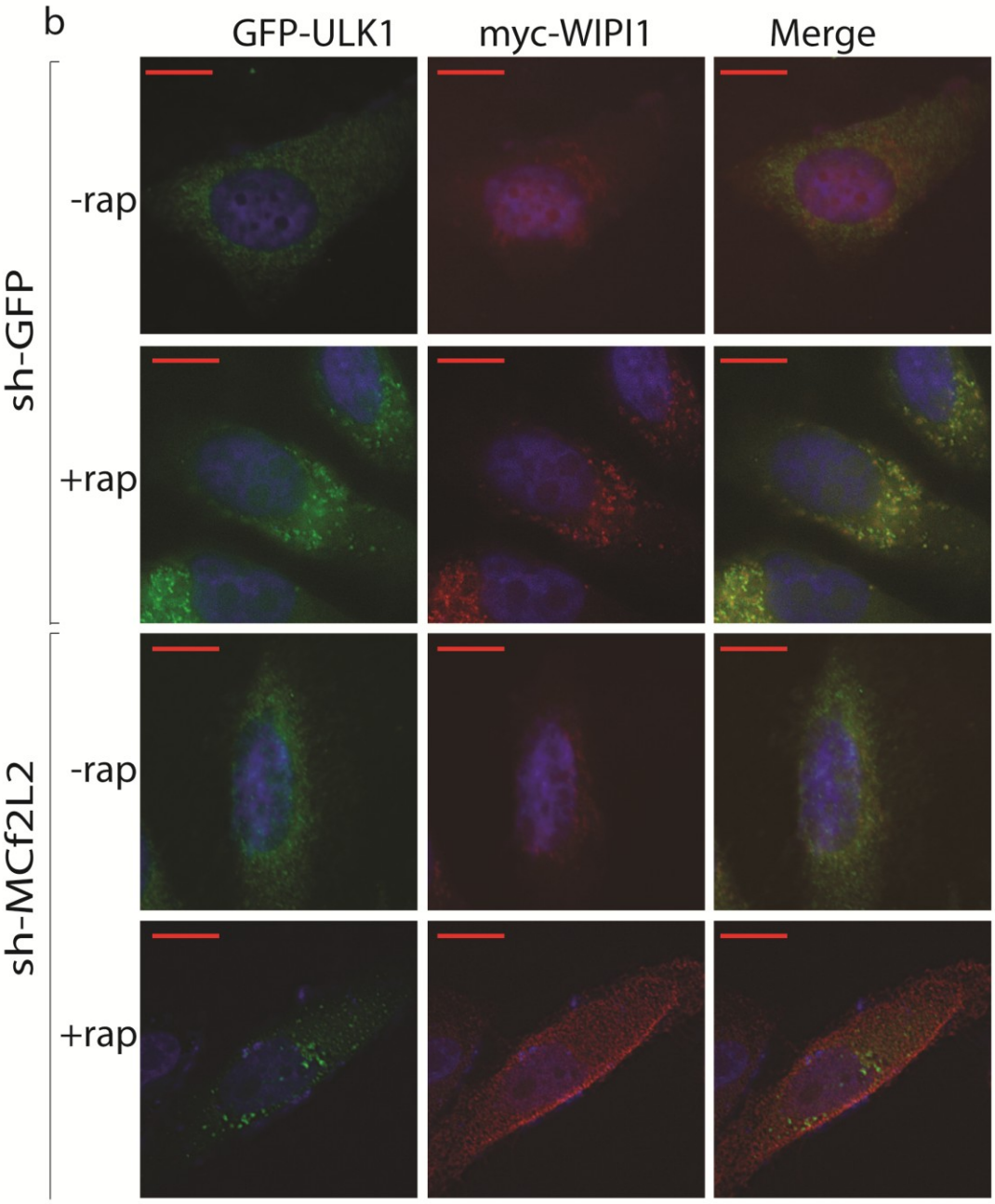
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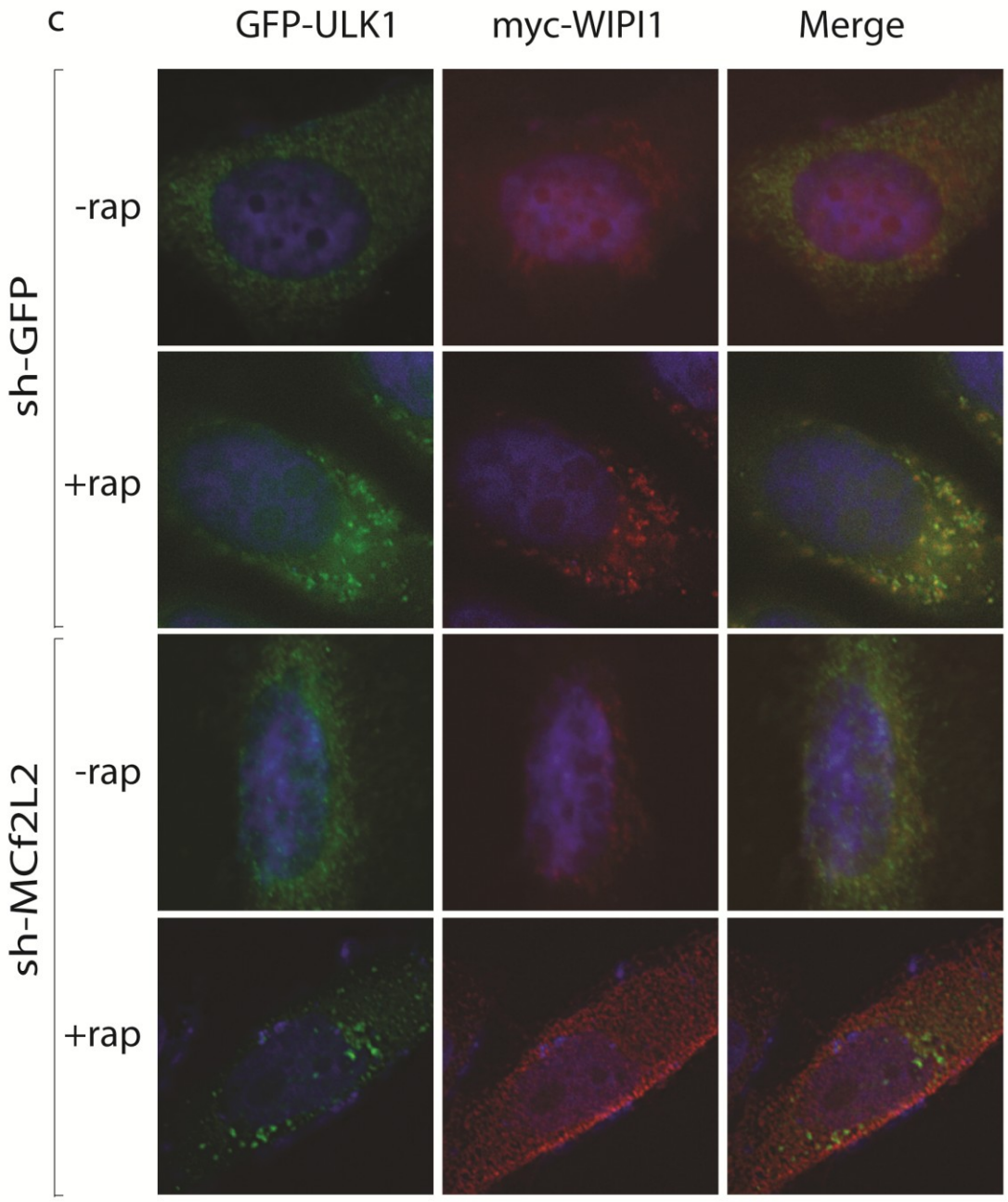
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+rap

d

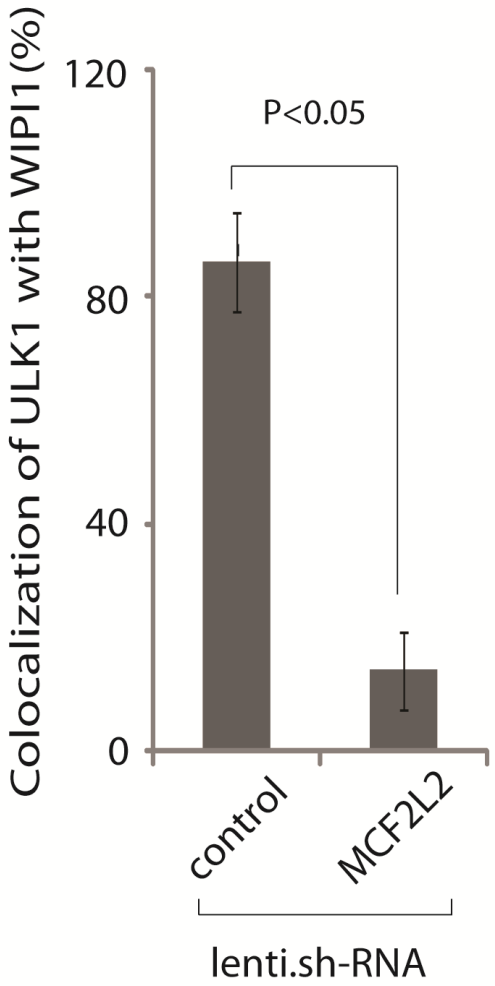

Colocalization of ULK1 with WIPI1 (%)
120
80
40
0
P<0.05
control
MCF2L2
lenti.sh-RNA

Figure 13. MCF2L2 is important for the WIPI-1 puncta formation.

(a) Silencing of MCF2L2 does not affect the formation of Atg14L puncta.

GFP-tagged Atg14L was expressed in HeLa cells treated with 100 nM rapamycin or DMSO as vehicle for 1 hr. GFP fluorescence in the cells was visualized by microscope. Nuclei were stained with DAPI (blue). Scale bar,10 μm.

(b) Knockdown of MCF2L2 inhibited the co-localization of ULK1 puncta with WIPI-1 puncta.

GFP-tagged ULK1 and myc-WIPI-1 were co-expressed in HeLa cells. Twenty-four hrs post-transfection, cells were treated with 100 nM rapamycin or DMSO for 1 hr. Cells were immunostained using Alexa647-conjugated myc antibody (red) and visualized by microscope. Colocalization of ULK1 puncta and WIPI-1 puncta is shown in yellow color. Nuclei were stained with DAPI (blue). Scale bar,10 μm.

(c) Higher magnification of images shown in (b).

(d) Quantification of the colocalization of ULK1 puncta and WIPI-1 puncta.

The numbers of ULK1 puncta and WIPI-1 puncta were counted in MCF2L2-silenced cells (n=18) or control cells (n=14). Percentage of ULK1 punta that are overlapped by WIPI-1 puncta relative to total ULK1 puncta was compared. Two sample Student t-test was conducted to assess the significance of difference between MCF2L2-silenced cells and GFP shRNA-control cells. When $p < 0.05$, the difference is considered statisically significant.

III. Potential function of MCF2L2 as a regulator of actin cytoskeleton

a. MCF2L2 has a partial GEF activity toward Rho small GTPases

To further understand the function of MCF2L2 in the regulation of autophagy processes, I have examined the possibility that MCF2L2 might function as a GEF for Rho small GTPases as MCF2 and MCF2L do. To test this possibility, I have used the well-established assay system that is based on monitoring the active state of Rho GTPases in cell lysate. I transduced HEK293T cells with a plasmid encoding HA-MCF2L2 and analyzed the amounts of GTP-bound Rho GTPases (RhoA, Rac1, and CDC42) versus their total amounts using the assay kit from Millipore. The active forms of the GTPases can specifically bind to the agarose beads with GST-tagged proteins containing the p21-binding domain (PBD, residues 67-150) of human p21-activated kinase-1 (PAK1). This bound form could be monitored by antibodies specific to Rho GTPases. I found that, compared to the control cells transduced by empty vector, cells transduced by MCF2L2-encoding plasmid exhibited higher levels of GTP-bound forms of Rho GTPases (Figure 14). The total amounts of Rho GTPases remained constant. Although the difference was not dramatic, this result supports a possible role of MCF2L2 as a GEF toward the small GTPases.

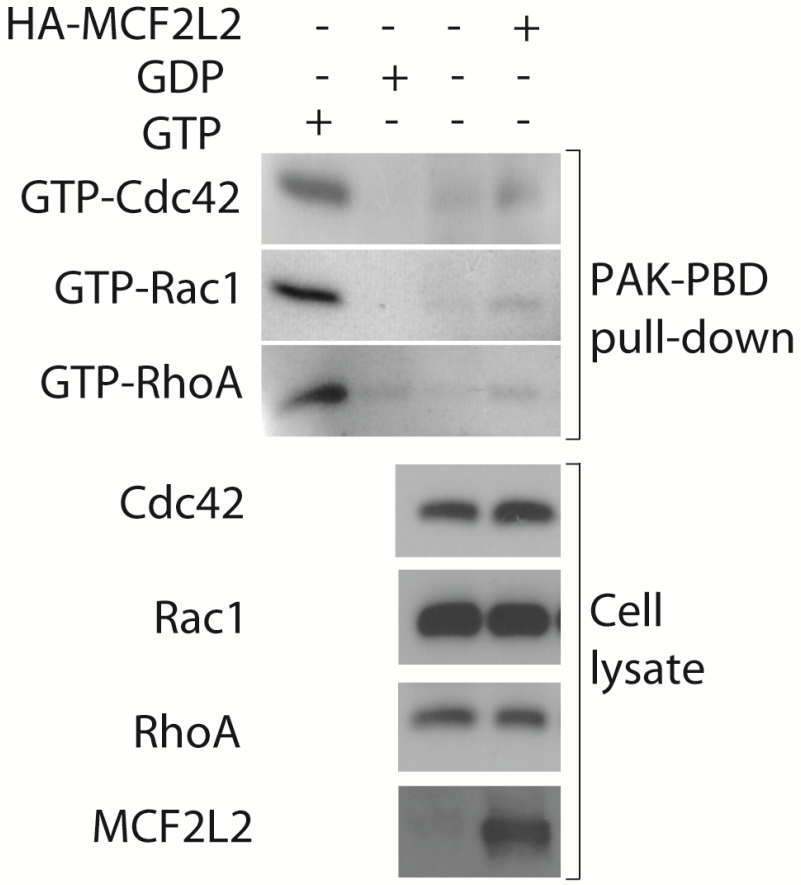


Figure 14. MCF2L2 shows a partial activity of GEF toward Rho small GTPases.

HEK293T cells were transfected with a plasmid encoding HA-MCF2L2 or empty vector as control. Forty-eight hrs after transfection, cell lysate was obtained and mixed with or without GTP or GDP. GTP-bound forms of Cdc42, Rac1, and RhoA were pulled down using PAK1-PBD agarose beads. The GTP-bound forms of GTPases on the beads and the total amounts of Cdc42, Rac1, and RhoA in the cell lysate were analyzed by Western blotting using Rho-GTPase antibodies.

b. MCF2L2 colocalizes with Arp3, a downstream effector of Rho GTPases.

Since Rho small GTPases are known as an important regulator of actin nucleation (Etienne-Manneville and Hall, 2002), I tested whether MCF2L2 might have an effect on actin nucleation. Actin nucleation is mediated by the Arp2/3 complex, which is regulated by Rho-GTPases (Liu et al., 2009b). I first investigated whether Arp3 localization is affected by nutrient-starved conditions. HeLa cells were transiently transduced by plasmids encoding GFP-Arp3 and cultured under Earle's Balanced Salt Solution (EBSS) medium for nutrient- and serum-starvation, the optimized condition to induce autophagy. GFP-Arp3 was monitored by microscope. Under complete cell culture medium, GFP-Arp3 was distributed throughout the cytoplasm (Figure 14). In EBSS medium, GFP-Arp3 formed puncta (Figure 15). This result suggests that the actin nucleation factor is somehow regulated by nutrient conditions. The GFP-Arp3 puncta colocalized with puncta of mCherry-MCF2L2 tht was coexpressed with GFP-Arp3 under EBSS (Figure 15). The colocalization of MCF2L2 puncta and Arp3 puncta implies that MCF2L2 may have an important role in Arp2/3-regulated actin polymerization.

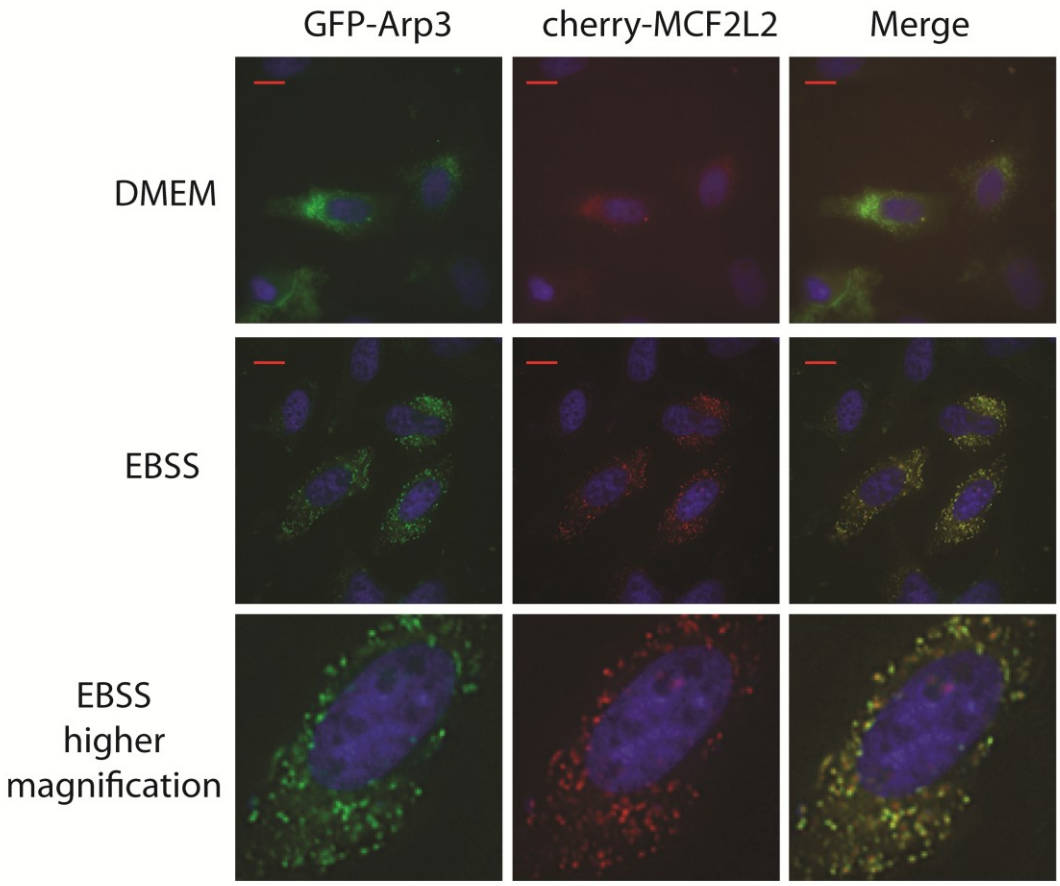


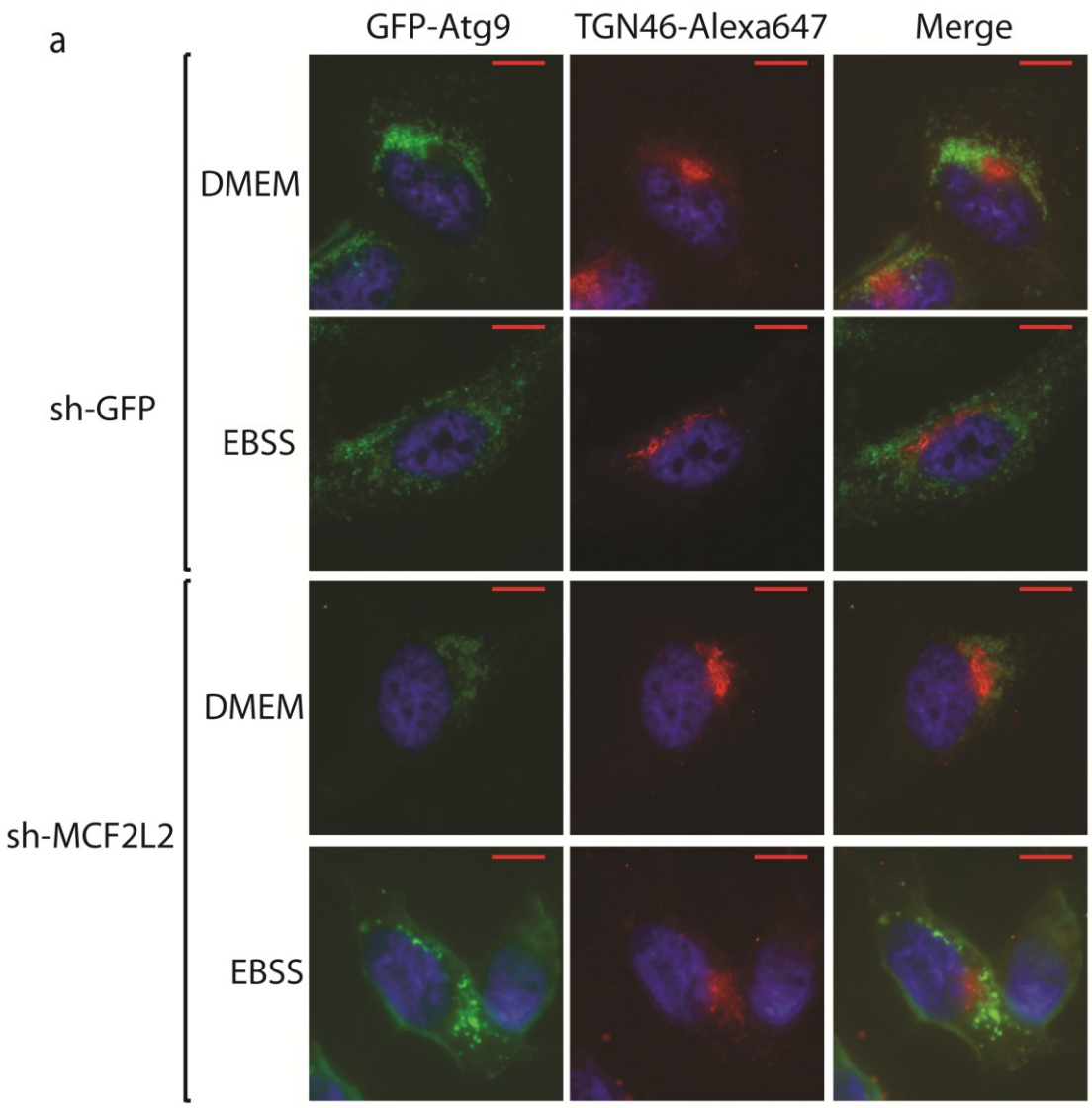
Figure 15. MCF2L2 puncta co-localizes with Arp3 puncta.

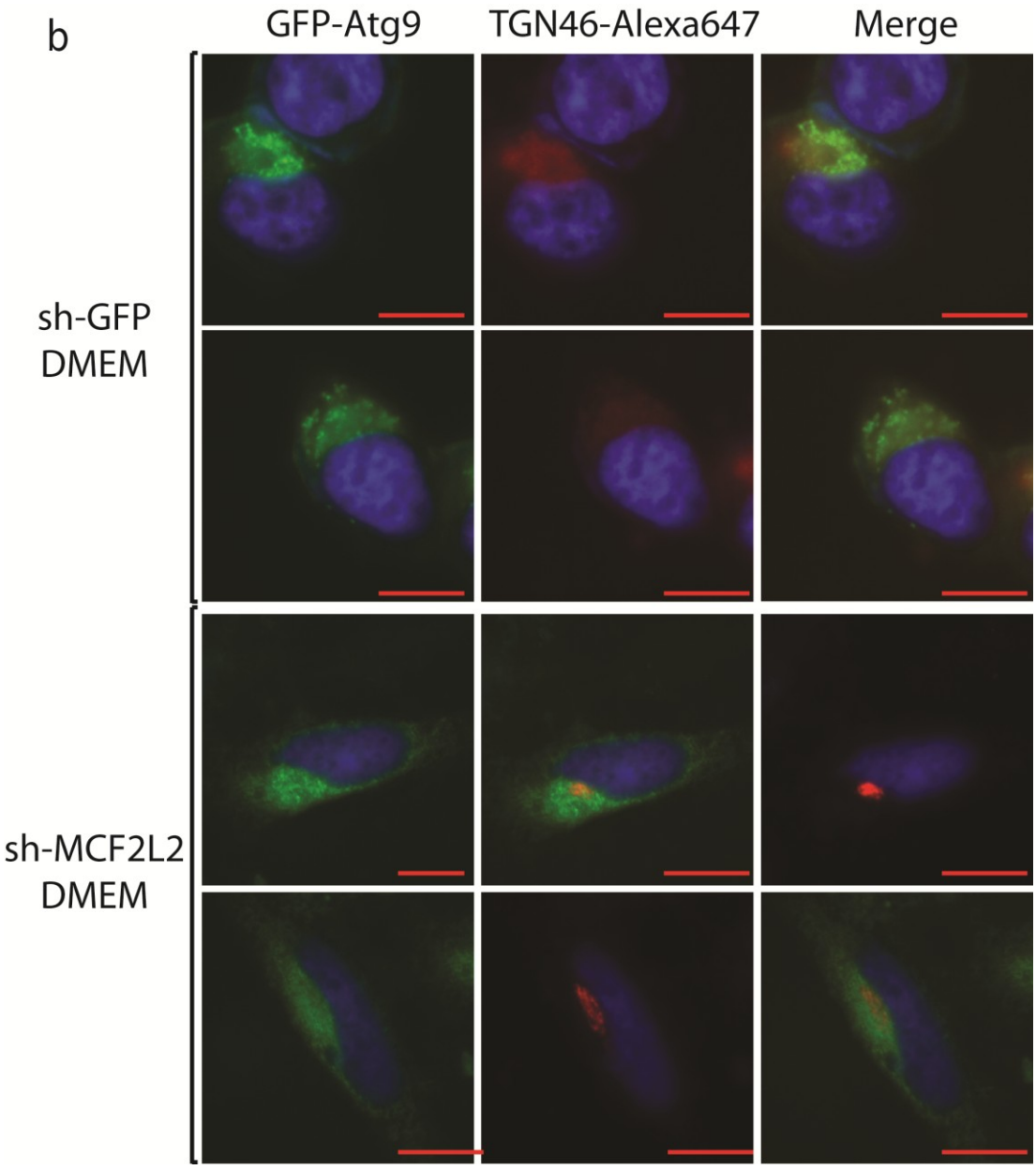
GFP-tagged Arp3 and mCherry-tagged MCF2L2 were expressed in HeLa cells. Cells were cultured under DMEM medium or EBSS starvation medium for 1 hr. GFP-Arp3 (green) and mCherry-MCF2L2 (red) were visualized by fluorescence microscope. Co-localization of Arp3 and MCF2L2 is shown in yellow color. Nuclei were stained with DAPI (blue). Scale bar,10 μm.

IV. MCF2L2 is important for the distribution of Atg9.

An important step in autophagy process that is possibly dependent upon actin polymerization is the re-distribution of Atg9 (Monastyrska et al., 2008). A study in yeast showed that Atg9 interacts with Arp2 during the trafficking of Atg9 between Golgi, endosome and the isolation membrane in autophagy (Monastyrska et al., 2008), implying that actin polymerization is involved in the trafficking of Atg9. The re-distribution has been proposed to be an important mechanism to provide membrane source for autophagosome (Sekito et al., 2009; Young et al., 2006). Studies in yeast and mammalian cells have shown that the trafficking of Atg9 may depend on the ULK1 complex (Reggiori et al., 2004; Young et al., 2006) and is likely regulated by the motor protein myosin and moves along actin filaments (Tang et al., 2010).

Thus, I considered the possibility that MCF2L2 might regulate the cellular distribution of Atg9. If MCF2L2 functions as a GEF for Rho GTPases and regulates Arp2/2 complex, MCF2L2 could affect Atg9 distribution by altering actin polymerization. To test this possibility, I compared the pattern of Atg9 distribution between MCF2L2-silenced cells and GFP shRNA-transduced control cells. To monitor the localization of Atg9, the shRNA-tranduced HeLa cells were transiently transduced by a plasmid encoding GFP-Atg9. Cells were incubated in either complete medium or EBSS starvation medium. The GFP-Atg9 as well as the trans-golgi compartment, which was monitored by using anti-TGN46 antibody, were visualized by microscope. Consistent with previous studies (Young et al., 2006), Atg9 localized to regions around TGN in complete medium. The TGN-localized Atg9 was re-distributed to a dispersed pattern in the cytoplasm in control cells under starvation. By contrast, MCF2L2 knockdown suppressed the distribution to the dispersed pattern with a large portion of Atg9 being retained in regions around TGN even in the starved condition (Figure 16). This result suggests that MCF2L2 is required for the distribution of Atg9 from TGN to cytoplasm during starvation.


a
GFP-Atg9
TGN46-Alexa647
Merge
DMEM
sh-GFP
EBSS
DMEM
sh-MCF2L2
EBSS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GFP-Atg9
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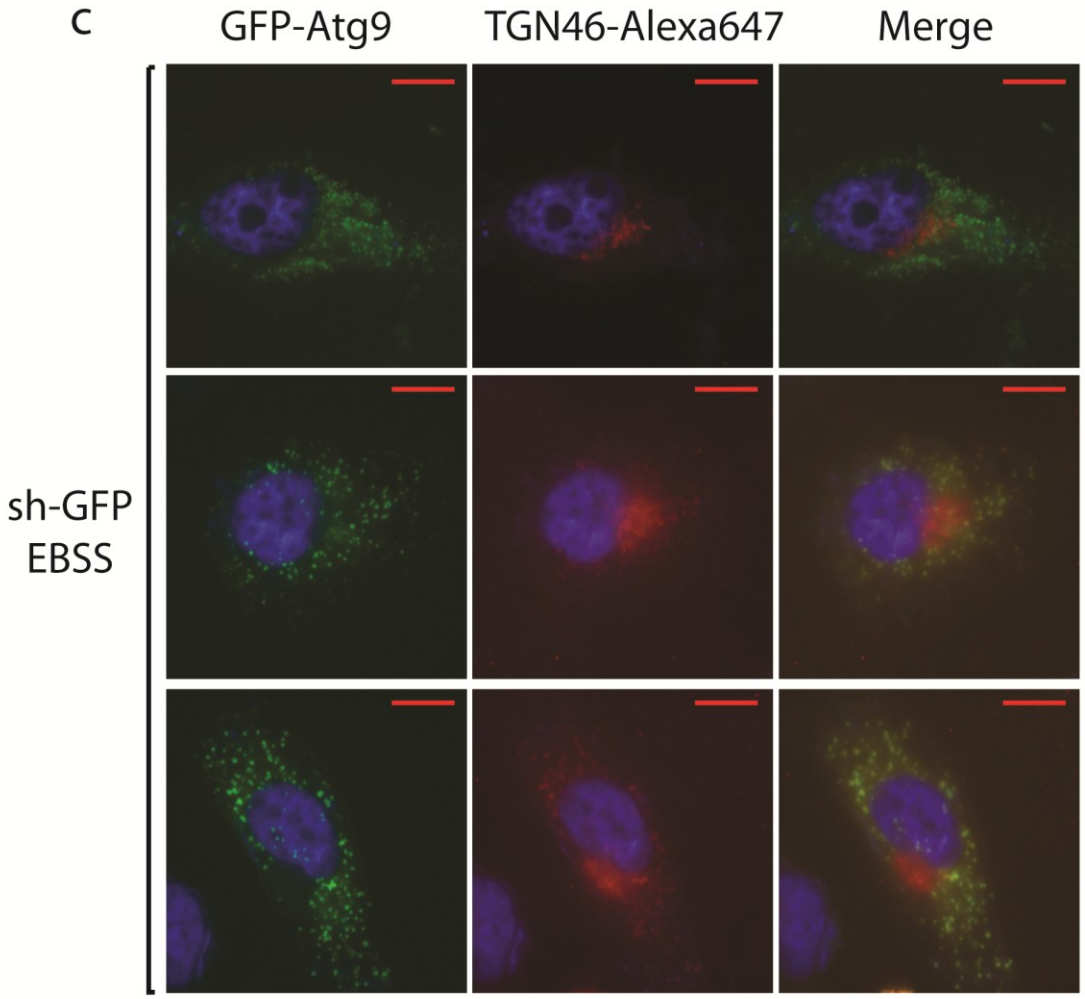
C
GFP-Atg9
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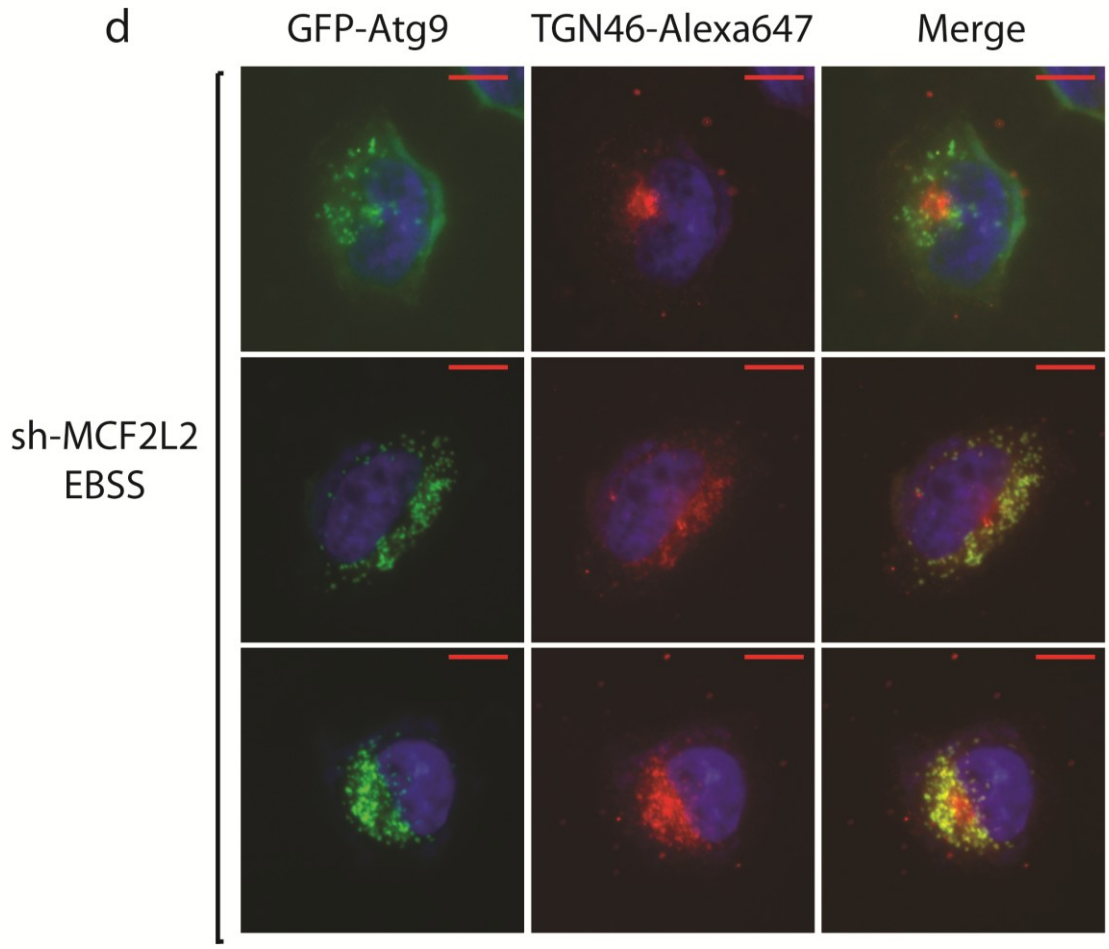
d
GFP-Atg9
TGN46-Alexa647
Merge
sh-MCF2L2
EBSS

Figure 16. MCF2L2 knockdown inhibited the distribution of Atg9 and retained Atg9 in the TGN under starvation.

(a) GFP-tagged Atg9 was expressed in MCF2L2-silenced HeLa cells and GFP shRNA-transduced control cells. Twenty-four hrs after transfection, cells were incubated in full DMEM medium or starvation medium (EBSS) for 1 hr. Cells were stained with trans-golgi network 46 (TGN46) antibody conjugated with Alexa647 (red) and were visualized by fluorescence microscope. Co-localization of Atg9 with the TGN is shown in yellow color. Nuclei were stained with DAPI (blue). Scale bar,10 μm.

(b)-(d) Additional images showing localization of Atg9 and TGN in EBSS-starved cells. Nuclei were stained with DAPI (blue). Scale bar,10 μm.

CHAPTER 3. DISCUSSION

My thesis work has led to the discovery of MCF2L2, a novel component of the ULK1 complex. My work has demonstrated that MCF2L2 interacts directly with Atg13 through its N-terminal region. The study has also revealed that MCF2L2 is important for autophagosome formation and autophagy flux. I have determined that MCF2L2 is required for WIPI-1 to localize to the isolation membrane, which is an important downstream event following the recruitment of the ULK1 complex and the Atg14L-containing PI3KC3 complex to the isolation membrane (Itakura and Mizushima, 2010). In addition, I have determined that MCF2L2 is important for the trafficking of Atg9, a process that provides membrane source for autophagosome (Orsi et al., 2012; Webber and Tooze, 2010; Young et al., 2006). My study has also provided supportive evidence that MCF2L2 might regulate actin polymerization. Consistent with the possible functions, previous studies have shown that Atg9 is tightly associated with Atg18, the yeast homolog of WIPI (Reggiori et al., 2004; Wang et al., 2001a) and the trafficking of Atg9 is regulated by motor proteins moving along actin filaments (Tang et al., 2010). Atg9 has also been proposed to participate in actin nucleation (Monastyrska et al., 2008). My study however could not clarify what is the exact mechanism through which MCF2L2 regulates autophagy. Nevertheless, this work has provided an important basis for the future study to elucidate the signaling pathway from the ULK1 complex to the autophagy induction process.

I. The architecture of the ULK1 complex associated with MCF2L2.

Among the major members in the ULK1 complex, FIP200 most likely serves as a scaffolding protein providing the platform for the ULK1 complex to assemble as a whole (Hara et al., 2008), but the function of Atg13 remains unknown. The binding of Atg13 to

the N-terminus of MCF2L2 implies that Atg13 may serve as a docking site for MCF2L2 to land on the FIP200 platform.

The C-terminal region of ULK1 may regulate the conformation and activity of ULK1 (Chan et al., 2009) on top of its function of mediating ULK-binding proteins (Chan et al., 2007; Gan and Guan, 2008). A study using a limited proteolytic analysis revealed that ULK1 C-terminal region appears to fold back with the N-terminal kinase domain to keep the protein in a "closed" conformation in a manner dependent upon autophosphorylation (Chan et al., 2009). A putative autophosphorylation site Ser1047 was identified at near the C-terminus of ULK1 (Dorsey et al., 2009). The proteins may bind to the C-terminal region of ULK1 directly (Atg13) or indirectly (MCF2L2) to have influence on the conformation and activity of ULK1.

II. MCF2L2 might regulate autophagy through the actin cytoskeleton.

Little is known about the mechanism by which WIPIs and Atg9 are translocated to the autophagy machinery. Based on my study, I propose two pathways through which MCF2L2 regulates the localization of WIPIs and Atg9. One pathway might involve MCF2L2 acting as a GEF for Rho small GTPases to facilitate the actin polymerization. The actin polymerization is required for Atg9-mediated autophagic membrane dynamics. An alternative pathway might involve MCF2L2 acting as a GEF to stimulate the movement of myosin along the actin filaments. Myosin serves as a cargo carrier to transport Atg9 and WIPIs to the origin of the isolation membrane formation (Figure 17).

In the first pathway, I propose that the GEF activity of MCF2L2 is important for actin polymerization. The cytoskeletons have been emerging to play an important role in the autophagy process (Monastyrska et al., 2009). While there have been some studies published on microtubules and their role in the vesicle membrane fusion in autophagy (Fass et al., 2006; Orsi et al., 2010), little is known about actin cytoskeleton in autophagy.

An important line of evidence has been provided by a recent study with respect to the function of actin cytoskeleton in the autophagy process. The local assembly of F-actin (the polymer form of actin) was found at the site of autophagosome-lysosome fusion (Lee et al., 2010b). This finding suggests that the autophagic membrane fusion might depend on actin polymerization. Based on the finding that MCF2L2 potentially contains a GEF activity (Figure 14) that can stimulate the actin nucleation catalyzed by the Arp2/3 complex, which appears to colocalize with MCF2L2 (Figure 15), I hypothesize that MCF2L2 might play an important role in the actin polymerization required for the formation of isolation membrane or for the fusion of autophagosomal membranes. Generation and expansion of the isolation membrane may require cytoskeletal re-organization fueled by actin polymerization in the proximal region, and this may explain that MCF2L2 knockdown possibly interferes with isolation membrane generation, as suggested by the abolished puncta formation of the isolation membrane marker WIPI-1 (Figure 13).

Another potential link of MCF2L2 to autophagy appears to be the regulation of Atg9 trafficking. In yeast, Atg9 was shown to interact with Arp2 during its trafficking among the autophagosome membrane sources (Monastyrska et al., 2008). Atg9 interacts dynamically with isolation membranes and autophagosomes but without being physically incorporated into the autophagosomal membranes (Orsi et al., 2012). Therefore, Atg9 is thought to deliver membranes for the biogenesis of autophagosome. The interaction between Atg9 and Arp2 probably indicates that the delivery of membranes by Atg9 might depend on active actin polymerization. MCF2L2 may be responsible for the activation of the actin polymerization through its GEF activity. The knockdown of MCF2L2 interferes with Atg9 translocation may suggest that Atg9 is involved in actin polymerization.

While the autophagic membrane dynamics may be coupled with actin polymerization within a local region, the protein factors required in the membrane biogenesis need to travel a longer distance in the cell. WIPIs were found to translocate from the cytosol to neonascent isolation membrane when autophagy is induced (Itakura

and Mizushima, 2010; Polson et al., 2010). In yeast, the WIPI homolog Atg18 was found to form a complex with Atg2 as an effector of PI3P (Obara et al., 2008). Atg18 also interacts with Atg9 in yeast to mediate membrane transportation (Reggiori et al., 2004; Wang et al., 2001a). The mammalian WIPIs are also effectors of PI3P (Polson et al., 2010), while its interaction with Atg9 has not been reported and how WIPIs facilitate the isolation membrane formation remains clear. Atg9 cycles between the TGN and the cytoplasma in autophagy (Young et al., 2006). Atg9 is thought to be a courier to deliver membrane sources for isolation membrane formation (Orsi et al., 2012; Young et al., 2006). The transport of Atg9 in yeast is dependent on the Atg2-Atg18 complex (Reggiori et al., 2004; Wang et al., 2001a) and the Atg1-Atg13-Atg17 complex (Reggiori et al., 2004; Sekito et al., 2009). In mammalian cells, whether Atg9 relocalization is dependent on ULK1 remains unclear (Orsi et al., 2012; Young et al., 2006). According to my study, it is possible that MCF2L2 plays a role in mediating the ULK1 complex function in the regulation of Atg9 trafficking.

A recent study suggested that the translocation of Atg9 relies on the movement of myosin along the actin filaments (Tang et al., 2010). Myosin is regulated by ROCK, a Rho-GTPase effector, and therefore it is subject to the regulation by Rho-GEF. A tentative model is that MCF2L2 activates RhoA and subsequently ROCK. The regulation of myosin by ROCK carries Atg9 along the actin filaments. This model is consistent with the result that MCF2L2 knockdown resulted in retaining of Atg9 on TGN in starvation induced autophagy (Figure 16). I also observed that knockdown of MCF2L2 had little effect on ULK1 and Atg14 recruitment to isolation membrane but it blocks the recruitment of WIPI-1 to the isolation membrane (Figure 12 & 13). It is possible that MCF2L2 is required for PI3P to regulate WIPIs, or MCF2L2 can directly regulate the transport of WIPIs. There has not been any report showing a link between Rho GEF and WIPIs. Given the physical interaction between Atg9 and Atg18 in yeast, MCF2L2 could also regulate WIPI translocation through the ROCK-myosin pathway. The yeast Atg2 forms a complex with Atg18 and regulates Atg9 (Obara et al., 2008). Mammalian Atg2

has recently been identified (Velikkakath et al., 2012). It would be interesting to investigate the possibility that the mammalian Atg2-WIPI complex is associated with Atg9.

III . A possible function of MCF2L2 in the formation of isolation membrane

My study has revealed that ULK1 induces phosphorylation of MCF2L2 in cells and can directly phosphorylate MCF2L2 in vitro (Figure 11c). Although these data are not sufficient to define MCF2L2 as a substrate of ULK1, it is highly likely that the MCF2L2 phosphorylation may be mediated by ULK1 given their interaction and functional connection. Possibly, the phosphorylation of MCF2L2 by ULK1 might play an important role in the stimulation of the ULK1 complex in the formation of the isolation membrane. Whether MCF2L2 is a substrate of ULK1 needs further investigation such as identification of phosphorylation sites. I attempted to identify phosphorylation sites by isolating MCF2L2 and analyzing it by mass spectrometry but failed to identify any phosphorylation sites. The reasons for the inability to identify phosphorylation sites include that the experiment may need to be optimized to enrich the phosphorylated protein and to increase the sensitivity of the mass spectrometry to identify the sites. Due to these technical complications, I could not include this work in my thesis.

At the initial stage of the autophagy process, ULK1 and Atg14L simultaneously gather in punctuate forms and locate to the isolation membrane (Itakura and Mizushima, 2010; Matsunaga et al., 2009). In mammalian cells, ULK1 is required for the formation of Atg14L puncta (Itakura and Mizushima, 2010). The formation of ULK1 puncta and Atg14L puncta is not blocked by wortmannin, the chemical inhibitor of PI3KC3. This supports the model that the ULK1 complex acts upstream of the Atg14-containing PI3KC3 complex in autophagy induction. In my study, the knockdown of MCF2L2 did not affect the puncta formation of ULK1 or Atg14L, indicating that MCF2L2 might not

be essential in ULK1-regulated PI3KC3 complex recruitment. However, knocking down of MCF2L2 interfered with the aggregation of PI3P effector, WIPI-1, and other downstream events in autophagy, such as the conversion of LC3-I to LC-II, autophagosome formation, and p62 degradation. These findings suggest that MCF2L2 may function downstream of the recruitment of PI3KC3 complex. The kinase-dead form of ULK1 is still recruited to the isolation membrane (Chan et al., 2007; Chan et al., 2009). Therefore, I speculate that MCF2L2 is recruited to the isolation membrane together with the ULK1 complex and the Atg14-PI3KC3 complex, and is subsequently phosphorylated by ULK1. Its GEF activity then regulates downstream events such as WIPI recruitment and autophagosome formation. To demonstrate this proposed model, it is essential to test the MCF2L2 mutant where a key residue in the GEF domain is mutated.

The function of MCF2L2 as a GEF toward small GTPases and actin polymerization regulator still remains ambiguous. A possible link for the putative MCF2L2 function in autophagy induction might be related to the regulation of the Exocyst complex consisting of 8 subunits EXO1-8, formerly known as Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70, and Exo84 (Bodemann et al., 2011). Exocyst has been identified as a binding complex of the ULK1 complex, providing important insight into the mechanism through which the ULK1 complex forms puncta tructure upon activation of autophagy and how it signals to the PI3KC3 complex (Bodemann et al., 2011). Nutrient deprivation activates Ras-like small GTPase RalB, which localizes to nascent autophagosomes, and RalB then activates its effector protein and Exocyst component Exo84 to promote autophagosome formation. The Exocyst complex was proposed to play a scaffolding role to allow for the interaction between ULK1 complex and the Beclin1-VPS34 PI3KC3 complex (Bodemann et al., 2011). It is possible that MCF2L2 regulates cytoskeleton rearrangement and thereby supports the scaffolding function of Exocyst.

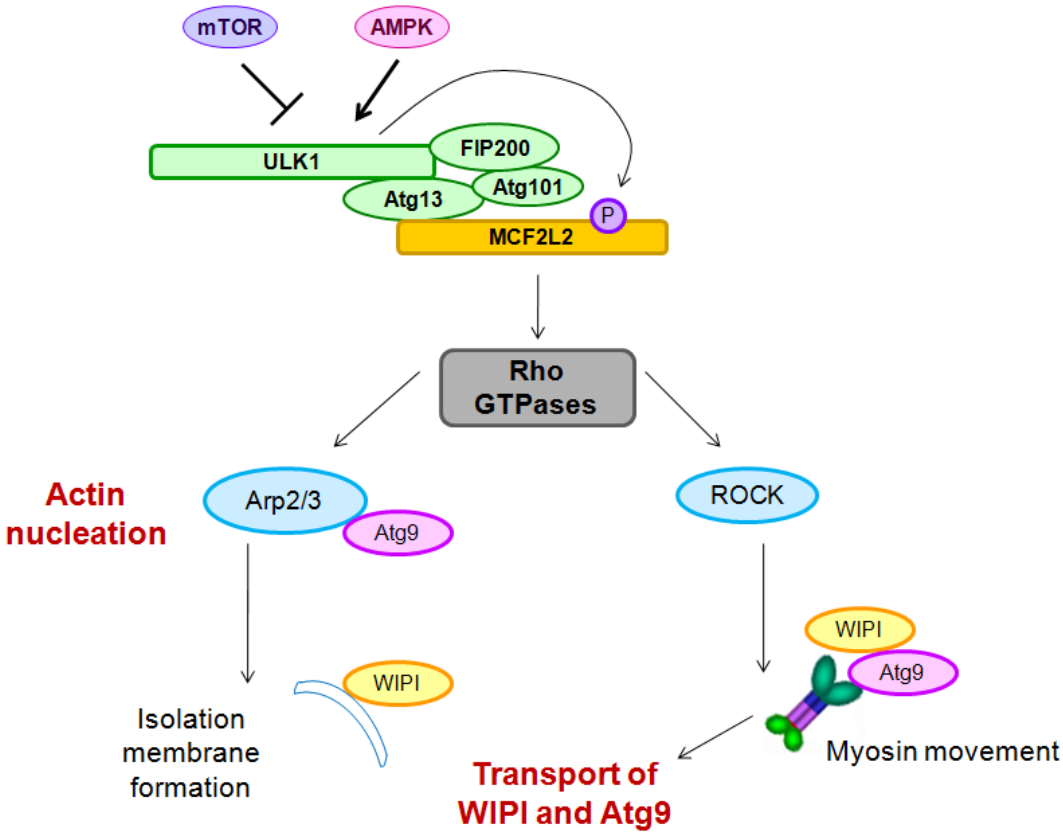

Figure 17. Proposed models for the regulation of MCF2L2 on autophagy through the cytoskeleton.

MCF2L2 was identified as a component of the ULK1 complex. It binds to Atg13 via its N-terminal region and to the C-terminal region of ULK1 via Atg13 (Figure 10). ULK1 potentially phosphorylates MCF2L2 (Figure 11). As part of the ULK1 complex, MCF2L2 is proposed to act as a GEF for Rho GTPases (Figure 14). The Rho GTPases activates Arp2/3 and actin polymerization. MCF2L2 may participate in the isolation membrane formation through its stimulation of actin nucleation (Figure 15). MCF2L2 may also exert its regulatory effects on the transport of Atg9 (Figure 16) and WIPI-1 (Figure 13) through the ROCK-myosin pathway.

IV. Summary

The yeast two hybrid screen for Atg13 binding partners led to identification of MCF2L2 as a novel component of the ULK1 complex. MCF2L2 directly interacts with Atg13 through its N-terminal region. MCF2L2 binds to the C-terminal region of ULK1 via Atg13. MCF2L2 plays an important role in the autophagosome formation and autophagy flux and it is a potential substrate of ULK1. MCF2L2 is not important for the formation of ULK1 puncta and Atg14L puncta but important for WIPI-1 recruitment to Atg14L puncta or the isolation membrane. Thus, MCF2L2 appears to play a role in the regulation of the process downstream of the ULK1 complex and the Atg14L-PI3KC3 complex. MCF2L2 is proposed to be a GEF for Rho small GTPases and it colocalizes with actin nucleation factor Arp3, one of the targets of Rho GTPases. The role of MCF2L2 in the regulation of actin cytoskeleton may contribute to the autophagic membrane trafficking mediated by Atg9 through the ROCK-myosin pathway. Although my study could not clarify the exact mechanisms by which MCF2L2 regulates autophagy induction, it provided the molecular basis for better understanding the mechanism for the recruitment of WIPIs and the trafficking of Atg9.

CHAPTER 4. PERSPECTIVES

a. How the MCF2L2-containing ULK1 complex contributes to the formation of the isolation membrane.

A fundamental but yet un-answered question in the field of autophagy is how the membrane source of autophagosome is recruited in response to the autophagy induction signals in a synchronized manner to generate the isolation membrane. Both *de novo* biogenesis and preexisting organelles might contribute to the membrane source of autophagosome (Weidberg et al., 2011). The multiple organelles include, including ER, mitochondria, Golgi and plasma membrane, have been proposed to provide membrane source to the formation of the isolation membrane. The finding of MCF2L2 as a component in the ULK1 complex suggests two possible contributions of the MCF2L2-containing ULK1 complex in the isolation membrane formation. MCF2L might regulate actin polymerization at the site of membrane biogenesis. As a consequence, MCF2L2 might facilitate the myosin movement on actin filaments to transport Atg9-containing membrane vesicles. To clarify the mechanisms, we propose several studies for the future works.

First, it will be necessary to identify the phosphorylation sites of MCF2L2 that are mediated by ULK1. Whether the phosphorylation site lies in the Rho-GEF or PH domain may imply if ULK1 regulates the Rho-GEF activity of MCF2L2. Determining the functions of the phosphorylations is anticipated to greatly improve understanding the mechanism by which ULK1 regulates the formation of the isolation membrane. Second, the function of MCF2L2 as a GEF toward small GTPases will need to be further clarified. I found that MCF2L2 has a moderate effect on the activation of Rho GTPases (Figure 14). Such a moderate effect might indicate that the tested small GTPases are not the *in vivo* substrates of MCF2L2. Alternatively, MCF2L2 might have only a weak activity as a GEF and it might have non-GEF functions. Sequence alignment between MCF2L2, MCF2L and MCF2 revealed that there is an insert of amino acids 772-794 between the DH domain and the PH domain only in MCF2L2 (Figure 8b). The insert might affect the

activity of MCF2L2 or its specificity in recognition of substrates. It will be interesting to test if deletion of the insert affects the activity of MCF2L2 in the autophagy pathway. With this regard, a recent study has identified Rac3, a closely related isoform of Rac1 and Rac2, as a negative regulator of autophagy (Zhu et al., 2011). It is possible that MCF2L2 can specifically target Rac3 and regulate the activity of Rac3 negatively instead of positively. If Rac3 is the mediator of MCF2L2 regulation on autophagy (Figure 14), it would also explain why MCF2L knockdown did not have effects on autophagy. Further studies are also needed to test if the GEF activity of MCF2L2 if regulated by the induction of autophagy.

Third, the interaction of MCF2L2 and Arp2 will need to be further characterized through multiple alternative approaches. My study has only provided the colocalization of MCF2L2 puncta and Arp2 puncta under starvation (Figure 15). It will be interesting to investigate how their interaction is regulated by nutritional status through co-iummunoprecipitation analysis. Once this interaction is demonstrated through co-immunoprecipitation, the binding sites on those proteins can be identified. The identification of binding sites could lead us to explore further the functional significance of the interaction in the regulation of actin polymerization and thereby the formation of the isolation membrane. Fourth, it will be important to further clarify the exact mechanism by which MCF2L2 regulates the trafficking of Atg9-embedded membrane vesicle and the recruitments of WIPIs to the isolation membrane. It would be worthwhile to test the Atg2-WIPI complex in interaction with Atg9 and how MCF2L2 contributes to the interaction during the formation of the isolation membrane. Lastly, the signaling pathway from the MCF2L2-containing ULK1 complex to the ROCK-myosin machinery might need to be explored, since it would provide a novel insight into the mechanism underlying the mobilization of the autophagy proteins required for isolation membrane formation.

b. How MCF2L2 links to obesity-related diseases could be understood in terms of its role in autophagy regulation.

There have been three publications about MCF2L2, and all of the studies are population studies to identify genetic linkages of obesity and obesity-related diseases (Takeuchi et al., 2008; Zhang et al., 2010; Zheng et al., 2009a). Takeuchi F et. al. reported three single nucleotide polymorphisms (SNPs) (rs35368790, rs35069869, and rs684846) in the non-coding region of MCF2L2 gene that are associated with a higher risk of type II diabetes in a group of Japanese population (Takeuchi et al., 2008). Two of the SNPs were identified in introns and one of them was identified in the 3' untranslated region. The three SNPs belong to different linkage disequilibrium groups, which contain multiple alleles that are associated at a higher frequency than would be expected from a random formation of haplotypes. This indicates that the 3 SNPs might not have been inherited together due to the linkage disequilibrium in this population. Another study by Zheng et al. 2009 revealed that the same 3 SNP variants in MCF2L2 are associated with higher risk of polycystic ovary syndrome (PCOS) in a group of Chinese population (Zheng et al., 2009a). A third study by Zhang D et. al. 2010 reported that two of the above SNPs are in the intron region and one more coding variant are linked to risk of diabetic nephropathy in a group of European decendants (Zhang et al., 2010). Obesity is a prevalent characteristic among the patients with diabetes and diabetic nephropathy, and a major portion of patients with PCOS develop insulin resistance. Therefore, these three diseases are thought be obesity-related diseases.

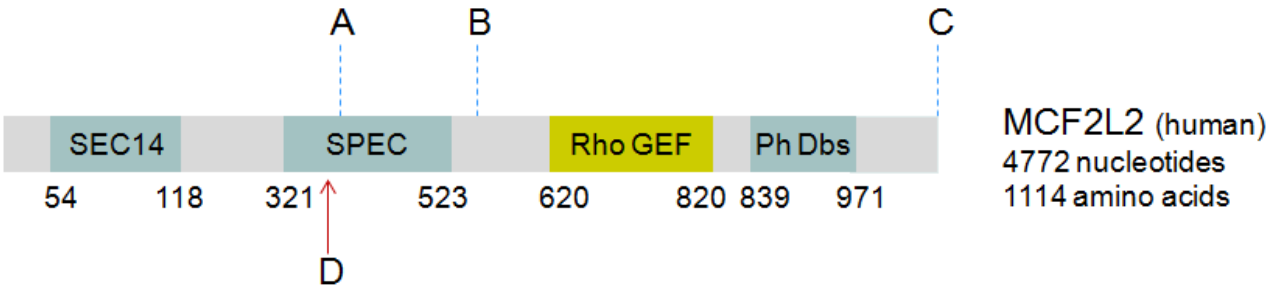

Figure 18. Disease-associated SNPs in the *MCF2L2* gene.

4 SNPs in *MCF2L2* gene (NCBI dbSNP) were reported to be associated to obesity-related diseases (Takeuchi et al., 2008; Zhang et al., 2010; Zheng et al., 2009a).

A. rs35368790—SNP in intron (major/minor allele: T/C)

B. rs35069869---SNP in intron (T/C)

C. rs684846---SNP in 3' untranslated region (C/T)

D. rs7639705---Coding variant (T/G, leucine359/isoleucine)

The studies above provide supportive evidence that MCF2L2's functions might be linked to the metabolic disease status. Knowing that MCF2L2 regulates autophagy, it is logical to speculate that the functional linkage of MCF2L2 to the metabolic diseases might be due to alteration of autophagy activity. There are emerging opinions on how autophagy activity is altered in diabetes or how autophagy can affect the disease progression (Jung and Lee, 2010). As an example, autophagy was found to participate in adipogenesis (Singh et al., 2009b), which contributes to insulin insensitivity. Besides the link between autophagy and adipogenesis, there are more avenues that can link MCF2L2 to diabetes. In pancreatic β islet cells, autophagy is thought to contribute to the secretion of insulin (Jung and Lee, 2010). Ras homolog family member Q, a member of the Rho family that is also called TC10 or RASL7A, is involved in the GTPase-dependent re-organization of cortical actin and induces translocation of intracellular vesicles containing glucose transporters upon insulin stimulation (Chiang et al., 2001).

PCOS is the most frequently occurring female endocrine disorder, with manifestations including multicystic ovaries as a distinctive feature, as well as dysfunctional follicular maturation and ovulation, and hyperandrogenism. A large portion of the patients also display metabolic abnormalities such as obesity and hyperinsulinism, and these patients carry increased risk of type II diabetes and cardiovascular diseases too (Shannon and Wang, 2012). There is very little evidence directly connecting autophagy to the origin of PCOS, but given the close relation of PCOS with diabetic metabolic disorders, it is fair to speculate that autophagy is implicated in the PCOS disease progress. The other disease that is linked to MCF2L2 SNPs, diabetic nephropathy is a main complication of diabetes. Podocytes (a subtype of kidney epithelial cells) were found to have high activity of autophagy, and deletion of Atg5 in mouse podocytes resulted in accumulation of proteinuria and glomerulopathy (Hartleben et al., 2010). This study suggests a protective role of autophagy against the metabolic stress in kidney cells.

The consistent association of MCF2L2 with the metabolic diseases implies that certain pathways dependent on MCF2L2 may be important in the obesity-related

diseases. Since MCF2L2 gene has not been identified in the mouse genome, it is impossible to study the role of MCF2L2 in the metabolic diseases using mouse models. Instead, it is feasible to investigate the role of autophagy in the obesity-related diseases in the future using mouse models targeting key autophagy genes .

MATERIAL AND METHODS

Materials

Anti- ULK1 (sc-10900 and sc-33182), tubulin (sc-12462), GAPDH (sc-25778) and 14-3-3 (sc-732) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p62 was purchased from BD Biosciences (no. 610832; San Jose, CA). Anti-hemagglutinin (HA) antibody (HA.11) was purchased from Covance (Berkeley, CA). Anti-Myc 9E10 mAb and rapamycin were purchased from EMD Biosciences (San Diego, CA). Anti-Atg13, ULK1, and MCF2L2 antibodies were generated by the Yenzym custom antibody service (South San Francisco, CA) from rabbits using glutathione S-transferase (GST)-tagged C-terminal (amino acid 250-1129) of protein (MCF2L2) or epitope peptides CETDLNSQDRKDLDK-amide and CDLGTFYREFQNPPQ-amide (Atg13) and MEPGRGGTETVGKFEFSC-amide (ULK1) as antigens as previously reported (Jung et al., 2009). Polyclonal anti-TGN-46 antibody was obtained from Serotec Inc. (Oxford, UK). ULK1-deficient MEFs were obtained from Dr. Kundu's group at St. Louis Children's hospital (Kundu et al., 2008).

Yeast two hybrid screen

Yeast expressing pGBT9-bait were grown in trp- dropout media. Human fetal brain library DNA (clontech) was transformed via LiAc/PEG method into prepared yeast cells. Cells were plated in trp-/leu-/his-/3mM 3-AT plates and incubated at 30 deg. C approximately 7-10 days. Primary isolates were restreaked on trp-/leu-/his-/3mM 3AT plates and grown several days for lacZ assays. Positive colonies that showed a color change in LacZ assays were picked for colony PCR. Alternatively, DNA was isolated from yeast and transformed in to bacteria for miniprep analysis and sequencing. Plasmids containing Gal4AD-cDNA library clones were recovered by transforming DNA into E. coli MH4 cells). Transformants were plated on M9 minimal medium lacking leucine but containing ampicillin. Plasmid DNA was isolated from bacteria and digested to confirm insert size. After sequencing, plasmid DNA was transformed into yeast strain AH109 in

combination with the original bait plasmid. Transformants were selected for growth on trp-/leu- plates, and streaked onto trp-/leu-/his-/3mM 3-AT plates to test for growth.

DNA constructions

Human MCF2L2 cDNAs were obtained from Katzusa Institute (KIAA08661, 2914 NTs) and Open Biosystems (Huntsville, AL; Image no. 5750039, 2101 NTs) and the two cDNA fragments were joined to generate the full length gene. Human ULK1 and Atg13 cDNA clones were obtained from Katzusa Institute in Japan. Human FIP200 cDNA clone was obtained from Open Biosystems (Image no. 3908134). MCF2L2, ULK1 and Atg13 fragments were obtained by PCR amplification and subcloned into HA- and myc-prk5 vector. The cDNAs for Atg9 and WIPI-1 were obtained from Open Biosystems. The GFP-Arp3 was kindly provided by Dr. Lorene Lanier in the Department of Neuroscience at the University of Minnesota.

Protein purification

The C-terminal fragments of MCF2L2 were expressed as GST-tagged forms in ArcticExpress bacterial cells (Stratagene, Agilent La Jolla, CA). Bacteria were cultured in LB medium for 4 hrs and induced with 1 mM isopropyl-1-thio-b-galatopyranoside (IPTG) followed by incubation of 16 hrs at 15°C. Bacteria were lysed in 50 mM HEPES (pH 7.5), 400 mM NaCl, 1 mM DTT, and 1 mM Phenyl-methyl-sulfonyl fluoride (PMSF) (Sigma Aldrich, St. Louis, MO). Cell lysate was centrifuged at 10,000 × *g* for 30 mins. The soluble supernatant fraction was again centrifuged for 40 minutes at 4 °C at 100,000 × *g* and the soluble extract was applied to a glutathione sepharose 4B column (GE Amersham, Piscataway, NJ). The GSH column retaining the bound proteins was washed with buffer (20 mM HEPES (pH 7.5), 300 mM NaCl, 1mM MgCl2, 1 mM DTT). GST-tagged proteins were treated with PreScission protease (GE Amersham, Piscataway, NJ) overnight at 4°C. The eluate, which contains GST-free proteins, was applied to SDS-PAGE and stained with Coomassie blue to determine the purity of proteins. The eluted

proteins were dialyzed against 20 mM HEPES (pH 7.5), 300 mM NaCl, 1mM MgCl2, 1 mM DTT to remove GSH and snap frozen in liquid nitrogen.

Cell culture and transfection

HEK293T, HeLa, and MEF cells were cultured in DMEM containing 10% of fetal bovine serum and 5% of penicillin/streptomycin at 37°C in 5% CO_2. For transient expression of proteins, cells were transfected with recombinant DNAs or short hairpin RNA (shRNA) plasmids using FuGENE 6 (Roche Applied Science, Indianapolis, IN) following the manufacturer's protocol. Cells were harvested 2d after transfection for coimmunoprecipitation assay or other biochemical or Western blot analysis.

Co-immunoprecipitation and Western Blotting

Whole cell extracts were prepared in a lysis buffer containing 0.3% Chaps or 1% Triton X-100 as described previously (Jung et al., 2009) and immunoprecipitated with antibodies described for each experiment. Precipitated proteins were washed four times with the lysis buffer, loaded onto 8% or 12% Tris-glycine gels, transferred onto polyvinylidene difluoride membranes (Bio-Rad, Richmond, CA), and detected with enhanced chemiluminescence Western blotting detection reagents (Perkin Elmer-Cetus, Norwalk, CT).

Lentiviral preparation and viral infection

Lentiviral shRNA transduction was performed as described previously (Jung et al., 2009). Briefly, the pLKO shRNA vectors encoding shRNAs were transfected into HEK293T with lentiviral packaging vectors pHR'8.2ΔR and pCMV-VSV-G using FuGENE 6. Viruses were collected 60 h after transfection. Target cells, such as HEK293T, HeLa, or MEF cells, were infected with the collected viruses for 4 h in the presence of polybrene. Lentiviral sh-RNA target sequences are 5'-

aacgtacgcggaatacttcga-3', 5'-gaatttggagctggaggat-3' and 5'-ccaggaaacttacagctca-3' for control, Atg13 and MCF2L2, respectively.

Western blot assay of autophagy flux and p62 level

The lentiviral shRNA-transduced HEK293T cells were treated with rapamycin or vehicle for 4 h in the presence or absence of pepstatin A (10 μg/ml) and E-64 (10 μg/ml), inhibitors of the lysosomal protein degradation (Sigma). Whole cell extracts were prepared in lysis buffer containing 1% Triton X-100. Cell lysates were run on SDS-PAGE, and proteins were transferred to PVDF membranes and probed with anti-LC3 mouse polyclonal antibody (Nanotools) and anti-p62 antibody (Bethyl Laboratories).

GST pull-down assay

The DNA constructs for GST-tagged MCF2L2N-terminal fragments (1-500 and 1-827) were cloned in the plasmid pGEX-6P-2 (Amersham Biosciences, Piscataway, NJ) and introduced into in ArcticExpress bacterial cells (Stratagene, Agilent La Jolla, CA). The GST fusion proteins were expressed by induction with 0.1 mM IPTG for 16 h. Bacteria were resuspended into a lysis buffer (50 mM Tris, pH 8, 100 mM NaCl, 1 mM DTT) containing 1 mg/ml lysozyme, and cells were sonicated on ice. Soluble fractions were collected and immobilized on 100 μl of glutathione-Sepharose 4B beads (Amersham Biosciences). GST alone or GST-tagged MCF2L2 N-terminal fragments were incubated with *bacterial*–purified Atg13 for 1 h at 4 °C. After washing the beads, the bound proteins were eluted by boiling samples for 10 min in the presence of electrophoresis loading buffer, and the amounts of Atg13 recovered with GST-tagged proteins were we analyzed by Western blotting.

***In vitro* kinase assay**

For ULK kinase assay, myc-tagged ULK1 were isolated by immunoprecipitation using anti-myc antibody (Calbiochem, OP10) from HEK293T cells. As substrates,

MCF2L2 (1-500 and 1-827) were purified from bacteria as described above. The kinase reaction was performed in a buffer containing 50 mM HEPES, pH 7.5, 1 mM EGTA, 0.01% Tween 20, 10 mM $MnCl_2$, 2.5 mM DTT, 100 μM ATP, 1 μg substrate, and the trace [γ-^{32}P]ATP for 30 and 60 min at 30°C. All the kinase reactions were stopped by adding 5× SDS-sample loading buffer.

Microscopic analysis of autophagosome formation

The lentiviral shRNA-transduced HeLa cells were seeded onto glass coverslips. Medium was refreshed with DMEM containing 10% fetal bovine serum after 24h, and autophagy was induced by 100 nM rapamycin or vehicle (DMSO) for 4 h in the presence of pepstatin (10 μg/ml) after an additional 24 h. Cells were fixed with formaldehyde, permeabilized using methanol and 1% Triton X-100, and stained with 4, 6-diamidinophenylindole (DAPI) and anti-LC3 antibody (Nanotools). Stained cells were visualized under Deltavision deconcolution microcope using 40X lense (Applied Precision, Inc. Issaquah, Washington). For visualization of MCF2L2, Arp3, WIPI, Atg9, and TGN-46, the corresponding GFP- or Cherry- constructs were transfected in the cell. Alexa fluorescent signal conjugated secondary antibodies were used to stain endogenous proteins.

Rho small GTPase activation assay

HEK293T cells were transfected with empty vector or a plasmid encoding HA-tagged MCF2L2. Cell lysates were collected and 500 μg of protein was used for the Rho small GTPase activity assay (Millipore, Rac1/Cdc42 activation kit cat# 17-441; RhoA activation kit cat# 17-294**).** GTP and GDP were added to the lysates as positive and negative controls. The agarose beads retaining GST tagged PBD (p21-binding domain containing residues 67-150 of human p21 PAK1) were used to selectively precipitate the active (GTP-bound) form of the Rho small GTPases. The precipitated small GTPase was then detected by Western blot using antibodies to Rho GTPases.included in the kit.

Statistical analysis

In the comparison of percentage of ULK1 punta overlapped by WIPI-1 puncta in MCF2L2-silenced cells vs. GFP control cells, Student t-test was used to detect the significance of difference. No outliers were excluded. $P<0.05$ was used to define statistical significance.

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